ISOLATION, MOLECULAR IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY PROFILING OF *ESCHERICHIA COLI* O157:H7 STRAIN ISOLATED FROM ANIMAL-ORIGIN FOODS IN HAWASSA AND YIRGALEM, SIDAMA, ETHIOPIA.

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HAWASSA, ETHIOPIA

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ADVISOR'S APPROVAL SHEET

This is to certify that the thesis entitled “Isolation, Molecular Identification and Antimicrobial Susceptibility Profiling of *Escherichia coli* O157:H7 Strain Isolated from Animal-Origin Foods in Hawassa and Yirgalem, Sidama, Ethiopia” submitted in partial fulfillment of the requirements for the degree of Master's with specialization in Veterinary Microbiology, the Graduate Program of the Department of Veterinary Medicine, and has been carried out by Workagegn Israel Asale ID. No, GPVeMi R/0005/14, under my supervision. Therefore I recommend that the student has fulfilled the requirements and hence hereby can submit the thesis to the department.

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We, the undersigned, members of the Board of Examiners of the final open defense by Workagegn Israel Asale have read and evaluated his thesis entitled “Isolation, Molecular Identification and Antimicrobial Susceptibility Profiling of *Escherichia coli* O157:H7 Strain Isolated from Animal-Origin Foods in Hawassa and Yirgalem, Sidama, Ethiopia” and examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial fulfillment of the requirements for the degree.

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DEDICATION

This thesis is dedicated to my beloved wife, Deborah Loha, whose unwavering support, love, and encouragement have been my strength throughout this journey.

DECLARATION

I hereby declare that this thesis, entitled "Isolation, Molecular Identification and Antimicrobial Susceptibility Profiling of *Escherichia coli* O157:H7 Strain Isolated from Animal-Origin Foods in Hawassa and Yirgalen, Sidama, Ethiopia" is my original work and has not been submitted to any other institution for any degree or qualification. All sources of information used in this research have been duly acknowledged.

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This MSc thesis has been submitted for examination with my approval as thesis advisor.

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I. ABBREVIATIONS

BPW	Buffered Peptone water
EHEC	Enterohemorrhagic *Escherichia coli*
ESBL	Extended Spectrum Beta Lactamase
HUS	Hemolytic Uremic Syndrome
LPS	Lipopolysaccharides
MDR	Multi drug resistance
PCR	Polymerase Chain Reaction
SIM	Sulphide Indole Motility
SMAC	Sorbitol MacConkey agar
STEC	Shiga toxin producing *Escherichia coli*
Stx	Shiga toxin
TBE	Tris/Borate/ Ethylene diamine tetraacetic acid,
TSB	Trypton soya broth
TSI	Triple sugar Iron

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VI. ABSTRACT

Escherichia coli O157:H7, an important foodborne pathogen, posing serious public health concern globally. This study conducted from December 2022 to July 2023 with the aim to isolate, molecularly identify and evaluate the antimicrobial susceptibility profiles of *Escherichia coli* O157:H7 isolated from animal-origin foods including beef, raw milk and fish in Hawassa and Yirgalem towns of Sidama region, Ethiopia. In this study, a total of 298 different samples were collected and analyzed for isolation and identification of *Escherichia coli* O157:H7 using culture, biochemical tests and PCR analysis. PCR analysis showed that *Escherichia coli* O157:H7 was isolated from 3.7% of the total samples of which 5.6% and 6.25% were isolated from beef and fish samples, respectively, and no *Escherichia coli* O157:H7 isolate was observed from milk samples. The observed isolation rate among the different food type samples was statistically significant ($p = 0.036$). It was revealed that meat samples collected from butcher shops were found to be more (7.6%) contaminated by *Escherichia coli* O157:H7 than samples from abattoirs (3.4%) ($p = 0.445$). Regarding fish samples, contamination level was greater in Nile tilapia (8.1%) than Catfish. Similarly, higher (7.3%) contamination was observed from samples collected from Gudumalle fish landing site than other sites. Moreover, fish skin was the more (8.6%) contaminated organ than fish muscle (3.4%). Antibiotic susceptibility test demonstrated that all *Escherichia coli* O157:H7 isolates exhibited 100% susceptibility to ciprofloxacin, nalidixic acid, chloramphenicol, cephalothin, ceftazidime, ceftriaxone, tetracycline, and gentamicin whereas a 81.8%, 45.5% and 100%resistance was observed in amoxacillin, streptomycin and clindamycin, respectively. The multidrug resistance observed in 45.45% of isolates with the calculated MDR index of 0.27, and no extended-spectrum beta-lactamase (ESBL) producing isolates were detected. The findings highlight the need for stringent hygiene practices and effective monitoring of animal-origin foods to minimize the risk of public health threats.

Keywords: Antimicrobial susceptibility, Beef, *Escherichia coli* O157:H7, Fish, Milk

1. INTRODUCTION

1.1. Background and Justification

Escherichia coli (*E. coli*) was first described in 1886 by Dr. Theodor Escherich as *Bacterium coli commune*, isolated from the fecal matter of an infant patient (Escherich, 1886; Stanford *et al.*, 2007). The pathogen belongs to the family Enterobacteriaceae, which also include *Salmonella*, *Klebsiella*, *Proteus*, and *Enterobacter* species. *E. coli* is a Gram-negative, rod-shaped (2.0–6.0 µm in length and 1.1–1.5 µm wide bacilli) bacteria with rounded ends (Stevens *et al.*, 2014). It is facultatively anaerobic, non-spore forming, and usually motile due to the presence of peritrichous flagella (Kaper *et al.*, 2004).

Taxonomically the bacteria are classified under phylum Proteobacteria, class Gamma proteobacteria, order Enterobacterales, family Enterobacteriaceae and genus *Escherichia*. Within the species *E. coli*, numerous strains and variants exist, distinguished by differences in genotype, phenotype, and pathogenicity. *E. coli* can be classified in to different strains on the basis of serotype, virulence factors, biochemical properties, and genetic markers (Garrity, 2007).

Escherichia coli bacteria comprise harmless commensal as well as pathogenic variants causing both intestinal and/or extra intestinal infections in humans and animals (Leimbach *et al.*, 2013). Although the bacterium is said to be commensal, it is one of the most common human and animal pathogens as it is responsible for a wide range of diseases (Kaper *et al.*, 2004; Ishii and Sadowsky, 2008; Allocati *et al.*, 2013).

Pathogenic *E. coli* strains include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and others, with EHEC, particularly the *E. coli* O157:H7 serotype, gaining global attention for its role in foodborne illness outbreaks (Nataro and Kaper, 1998; Tarr *et al.*, 2005). *E. coli* O157:H7 was first recognized as a human pathogen in 1982 during outbreaks of hemorrhagic colitis, characterized by abdominal cramps,

bloody stools, and little or no fever (Riley *et al.*, 1983). It has since become one of the most significant foodborne pathogens globally (Lim *et al.*, 2010).

Cattle are primary reservoir of *E. coli* O157:H7, asymptomatically shedding the with feces and it is estimated that between 1% and 50% of healthy cattle carry and shed the bacteria in their feces at any given time (Dunn *et al.,* 2004; Cho *et al.,* 2006). Other domesticated animals including sheep, goats, pigs, and turkeys and wild animals have also been found to shed *E. coli* O157:H7 in their feces (Lim *et al.,* 2010). Contamination of food products is often linked to bovine fecal material, with improper handling during milking and slaughtering contributing to the contamination of dairy and meat products (Bélanger *et al.,* 2011; Bello *et al.,* 2015).

Milk contamination typically occurs due to direct exposure to feces, often as a result of poor hygiene practices during milking. Similarly, contamination of meat is a concern when animals are slaughtered under unsanitary conditions, compromising the safety and quality of the final product. Consumption of raw or undercooked animal products, such as unpasteurized milk or undercooked beef, remains the primary transmission route of *E. coli* O157:H7 to humans (Hamid *et al.*, 2018; Ababu *et al.,* 2020). Though *E. coli* O157:H7 does not occur in fish microbiota, it has been detected aquatic environments including through contamination due to improper aquaculture practices and environmental pollution raising concerns about food safety and public health (Guzmán *et al.*, 2004).

In Ethiopia, its presence in various food sources has raised significant public health concerns. Studies have shown contamination rates 2.4% in meat samples in Hawassa (Atinafae *et al.*, 2017), 12% in Addis Ababa (Hamid *et al.*, 2018), 8.9% in Bahir Dar city (Ayenew *et al*., 2021), and 9.1% in Ambo town (Tadese *et al*., 2021). In dairy products, contamination rates have been found 4.08% in different Central Ethiopia regions (Dejene *et al.*, 2022), 12% in Bishoftu (Bedasa *et al*., 2018), and 8.9% in Assela (Abunna *et al*., 2018), while fish samples from lakes Hawassa and Abaya revealed contamination rates of 2.3% (Tilahun and Engdawork, 2020) and 14.1% (Walelign *et al.*, 2022), respectively.

Antimicrobial resistance (AMR) is a significantly growing global health concern endangering the life of humans and animals (Ferri *et al.*, 2017; Almansour *et al.*, 2023). Infections and resistance originating in humans, animals, and farm environments will inevitably disseminate infection with resistant pathogens and genes in the wider environment (Woolhouse *et al.*, 2015; Lepper *et al.,* 2022).

The emergence of antimicrobial resistance (AMR) poses a growing global threat, with resistance in human pathogens closely linked to bacteria in livestock and the environment (Lepper *et al.*, 2022). The misuse of antibiotics in both human medicine and agriculture accelerates the development of resistant strains (Spellberg *et al.*, 2016). In Ethiopia, inappropriate antibiotic use is a significant issue, with many bacterial strains showing resistance to commonly used drugs, leading to multidrug resistance (MDR) (Muhie, 2019).

Studies in Ethiopia have found that *E. coli* O157:H7 bacteria from raw cow milk and beef are highly resistant to many antibiotics. For example, 92.8% to ampicillin (Haile *et al.*, 2022), 44.4% to tetracycline (Sebsibe and Asfaw, 2020), 84.7% to streptomycin (Bedasa *et al.*, 2018), 100% to cephalothin and 22.2% to chloramphenicol (Beyi *et al.*, 2017). Fish isolates were also resistant, with 100% resistance to ampicillin and cefoxitin (Tilahun and Engdawork, 2020). This increasing antimicrobial resistance emphasizes the need for better management of antibiotic use and stricter food safety protocols to mitigate the spread of resistant strains.

1.2. Statements of the Problem

Despite the growing concern about *E. coli* O157:H7 and antimicrobial resistance, there is a paucity of data on the occurrence and contamination rate of this pathogen in foods of animal origin in Ethiopia and specifically in the current study sites. Most existing studies were focused mainly on central part of Ethiopia, including Addis Abeba, Bishoftu, Holeta, Modjo, and Sululta, only very few in other parts of the country (Mesele and Abunna, 2019; Geresu and Regassa, 2021).

In Ethiopia, foods of animal origin are a key part of the local diet, yet they are often processed and sold under conditions that may not meet standard hygiene practices (Haileselassie *et al.*, 2013; Abebe *et al.*, 2020; Gutema *et al.*, 2021; Adem and Haramaya, 2022). This study addresses these critical gaps by investigating the occurrence, molecular detection and identification, and antimicrobial susceptibility of *E. coli* O157:H7 in animal-origin foods in the current study areas.

1.3. Objectives

1.3.1. General objectives

- To isolate, and molecularly identify *E. coli* O157:H7 and also to evaluate antimicrobial susceptibility profile of *E. coli* O157:H7 isolated from animal-origin foods in Hawassa and Yirgalem towns of Sidama region, Ethiopia.

1.3.2. Specific objectives

- To isolate, biochemically and molecularly identify *E. coli* O157:H7 from meat, milk and fish in Hawassa and Yirgalem, Sidama, Ethiopia.
- To determine antimicrobial susceptibility status of the isolated *E. coli* O157:H7 strains against commonly used antibiotics.

1.4. Research Questions

1. What is the prevalence of *E. coli* O157:H7 in animal-origin foods in the study area?
2. Are animal-origin foods intended for human consumption in the study area commonly contaminated with pathogenic *E. coli* O157:H7?
3. What is the antimicrobial susceptibility profile of *E. coli* O157:H7 isolates in the study area?
4. How prevalent are extended-spectrum beta-lactamase producing *E. coli* O157:H7 strains in meat, milk, and fish samples from the study area?

2. LITERATURE REVIEW

2.1. *Escherichia coli* O157:H7

Escherichia coli O157:H7 is so-named because it expresses the 157th somatic (O) antigen identified and the 7th flagellar (H) antigen (Lim *et al*, 2010). The chromosomal size of *E. coli* O157:H7 is 5.5 Mb and it shares 4.1 Mb backbone sequence with in all *E. coli* strains (Perna *et al.*, 2001). *E. coli* O15:H7 has emerged through four sequential events; acquisition of an stx2-containing bacteriophage, acquisition of *pO157* and the *rfb* region, acquisition of the stx1-containing bacteriophage, and loss of the ability to ferment D-sorbitol and loss of β-glucuronidase activity (Wick *et al.*, 2005; Lim *et al.*, 2010).

E. coli O157:H7 is a most prominent and well-studied *E. coli* strain that can cause severe foodborne illness (Singha *et al.*, 2023).It is known for producing a toxin called Shiga toxin, which can cause damage to the lining of the intestines, leading to bloody diarrhea, abdominal cramps, and in severe cases, kidney failure (Mead and Griffin, 1998). *E. coli* O157:H7 infections can have significant public health and economic impacts, including healthcare costs, productivity losses, and food recalls (Sajeena and Kalyanikutty, 2024).

2.2. Laboratory Characteristics of *Escherichia coli* O157:H7

Detecting and identifying *Escherichia coli* O157:H7 in laboratory settings typically involves several methods aimed at isolating the bacterium from clinical samples or food matrices and confirming its identity. It starts with culture-based methods**:** including enrichment of samples like milk, meat, feces, food, and water. These samples are initially enriched in selective media such as buffered peptone water and modified Tryptic Soy Broth (mTSB). The enrichment step promotes the growth of *E. coli* O157:H7 (Batt, 2014; Abdel-Aziz and Eid, 2024).

Following enrichment, samples are plated onto selective agar media, such as sorbitol MacConkey agar (SMAC) or cefixime potassium tellurite sorbitol MacConkey agar (CT-

SMAC). On SMAC, *E. coli* O157:H7 appears as colorless or pale colonies, unlike other *E. coli*strains which ferment sorbitol and produce pink colonies. Morphologically, it appears as Gram-negative rods under a microscope (Murray *et al.*, 1996; Kang and Fung, 1999).

E. coli O157:H7 are unable to ferment sorbitol within 24h and cannot hydrolyze 4-methylumbrelliferyl-D-glucuronide (as lacking β-glucuronidase enzyme) (Okrend *et al.*, 1990). In SMAC, the ability of *Escherichia coli* O157:H7 to ferment sorbitol is a key characteristic used for its selective identification (Kang and Fung, 1999). *E. coli* O157:H7 typically does not ferment sorbitol, resulting in colorless or pale colonies on SMAC (MacFaddin, 2000).

Escherichia coli O157:H7 possesses several distinctive characteristics that differentiate it from other strains of *E. coli* other than being SMAC negative. They also do not grow well at temperatures > 44.5 °C (Raghubeer and Matches, 1990; Beneduce *et al.*, 2003) and they are well known to produce of potent toxins, particularly Shiga toxins (Stx1 and Stx2). These toxins are responsible for the severe symptoms associated with *E. coli* O157:H7 infection, including bloody diarrhea and hemolytic uremic syndrome (HUS). So, *E. coli* O157:H7 has a relatively low infectious dose (10-100 CFU/g), meaning that a small number of bacteria can cause infection (Griffin, 1994; Tilden *et al.*, 1996).

Various biochemical tests are conducted to confirm the identity of *E. coli* O157:H7. Most biochemical reactions of *E. coli* O157:H7 isolates are typical of *E. coli*, with the exception of sorbitol fermentation and (β-glucuronidase activity) (MacFaddin, 2000). *E. coli* O157:H7 typically exhibits specific biochemical reactions consistent with its identification. *E. coli* O157:H7 are facultatively anaerobic and produce gas from fermentation of carbohydrates, as seen by acid and gas production from lactose at 37^0C. *E. coli* have positive result in methyl red reaction indicating mixed acid fermentation of glucose, but negative in Voges–Proskauer reaction (acetoin production) (Murray *et al.*, 1996).

They produce indole, but are unable to hydrolyze urea or grow in Møller's Potassium Cyanide (KCN) broth (demonstrating an inability to grow in the presence of cyanide). Furthermore,

production of hydrogen sulphide is not normally evident when *E. coli* are cultured on triple sugar iron (TSI) agar or Kligler's iron agar (KIA) (Percival and Williams, 2014). They also do not induce gelatin liquefaction through gelatinase activity and the majority of strains decarboxylate lysine, use sodium acetate, but do not grow on Simmons' citrate agar, where citrate is the sole carbon source (MacFaddin, 2000; Batt, 2014).

In case of serotyping, the serotype of *E. coli* O157:H7 is determined through antigenic characterization. *E. coli* O157:H7 possesses somatic antigen (O157) and flagellar antigen (H7) (Padhye and Doyle, 1992) and serotyping involves testing for the presence of these specific surface antigens (Deisingh and Thompson, 2004).

E. coli O157:H7 harbors several pathogenicity islands (regions of the genome associated with virulence) that encode for various virulence factors, including adherence factors and toxins. These genetic elements contribute to the bacterium's ability to cause disease (Batt, 2014). *E. coli* O157:H7 can also be characterized by the presence of virulence factors, particularly genes encoding for Shiga toxins (Stx1 and Stx2) by using different techniques. Among the most important virulence characteristics of *E. coli* O157:H7 is its ability to produce one or more Shiga toxins (also called verocytotoxins, and formerly known as Shiga-like toxins) (Law, 2000; Singha *et al.*, 2023).

Shiga toxin genes are genetic elements responsible for encoding the toxins produced by Shiga toxin-producing *Escherichia coli* (STEC) strains (Mead and Griffin, 1998).The two main types of Shiga toxins are Stx1 and Stx2, each with several subtypes (e.g., Stx1a, Stx1c, Stx2a, Stx2b, etc.) (Melton-Celsa, 2014). These genes are often located on temperate bacteriophages (viruses that infect bacteria), lysogenic bacteriophages, or prophages, which integrate into the bacterial chromosome. The expression of Shiga toxin genes can be regulated by various environmental factors, such as exposure to antibiotics, temperature changes, and the presence of specific nutrients (Schmidt, 2001; Karmali, 2018).

Detection of Shiga toxin genes in *E. coli* isolates is essential for the identification of potential STEC strains and the assessment of their pathogenicity (Wang *et al.*, 2024a). Various

molecular techniques, including PCR, DNA hybridization assays, and whole-genome sequencing, can be used to detect and characterize Shiga toxin genes in bacterial isolates (Parsons *et al.*, 2016; Bai *et al.*, 2019).

Molecular typing techniques such as pulsed-field gel electrophoresis (PFGE) and whole genome sequencing (WGS) are used for detection of *E. coli* O157:H7 with highest accuracy. Molecular methods are important part of confirmatory identification (Söderlund *et al.*, 2014). Polymerase chain reaction (PCR) is most commonly used molecular approach and it targets specific genes, such as the *eae* gene (intimin), *stx* genes (encoding Shiga toxins), and O157 antigen genes (e.g., *rfbE*), are used for rapid and sensitive detection of *E. coli* O157:H7 DNA in clinical and food samples (Paton and Paton, 1998; Parsons*et al.*, 2016).

Real-Time PCR (quantitative PCR) assays offer enhanced sensitivity and specificity for the detection and quantification of *E. coli* O157:H7 DNA. Multiplex quantitative PCR assays targeting multiple virulence genes and serotype-specific markers are commonly employed (Ibekwe and Grieve, 2003). Loop-mediated isothermal amplification (LAMP) assays provide a rapid and cost-effective alternative to PCR for detecting *E. coli* O157:H7 DNA under isothermal conditions, without the need for sophisticated equipment (Notomi *et al.*, 2000; Malabadi *et al.*, 2024).

2.3. Epidemiology of *Escherichia coli* O157:H7

The epidemiology of *Escherichia coli* O157:H7 encompasses various aspects related to the occurrence, distribution, transmission, and control of this pathogen. *E. coli* O157:H7 is one of the major serotypes of Shiga toxin-producing *E. coli* (STEC) that can cause foodborne illness. It is found worldwide and has been isolated from various sources, including cattle, other ruminants, foods, water, and environmental samples (Mead and Griffin, 1998).

Cattle are considered the primary reservoir for *E. coli* O157:H7, with the bacterium colonizing the gastrointestinal tract of healthy cattle (Wang *et al.*, 2017; Dixon *et al.*, 2020). Other ruminant species, such as sheep and goats, can also carry and shed the bacterium.

Additionally, wildlife and environmental reservoirs may contribute to the transmission of *E. coli* O157:H7 (MacDonald *et al.*, 1996; Doyle *et al.*, 2006).

2.4. Methods of transmission of *Escherichia coli* O157:H7

Escherichia coli O157:H7 can be transmitted through various routes, primarily through the ingestion of contaminated food, water, or contact with infected animals or environments. Consumption of food, like raw or undercooked meat and raw milk, contaminated with *E. coli* O157:H7 is one of the most common modes of transmission. Contaminated foods may include: undercooked ground beef: raw or undercooked ground beef is a frequent source of *E. coli* O157:H7 contamination (Riley *et al.*, 1983).

The bacterium can be present in the intestines of cattle and can contaminate meat during processing. Similarly, raw fruits and vegetables; fresh produce, such as leafy greens, sprouts, and melons, can become contaminated with *E. coli* O157:H7 through contact with animal feces, contaminated irrigation water, or during handling and processing (Singha *et al.*, 2023; Doyle *et al.*, 2006). Raw milk and unpasteurized dairy products can harbor *E. coli* O157:H7 if contaminated during milking or processing (Saeed, 2024; Silveira *et al.*, 2023).

Drinking water contaminated with *E. coli* O157:H7 or using contaminated water for washing produce or cooking can lead to infection. Contamination of water sources can occur through runoff from agricultural areas, sewage contamination, or inadequate water treatment (Saxena *et al.*, 2015) Direct or indirect contact with fecal matter from infected individuals or animals can lead to transmission of *E. coli* O157:H7. This can occur through poor hygiene practices, inadequate handwashing, or contact with contaminated surfaces or objects (Olsen *et al.*, 2002). *E. coli* O157:H7 can also spread from person to person, particularly in settings where hygiene is compromised or close contact occurs. This can include households, childcare facilities, schools, and healthcare settings (Bach *et al.*, 2002; Seto *et al*., 2007).

2.5. The Virulence Factors of *Escherichia coli* O157:H7

E. coli O157:H7 virulence factors are genetically driven and typically encoded by plasmids, bacteriophage, transposons, and pathogenicity islands. The ongoing evolutionary process has yielded to a highly versatile species that is capable of colonizing, multiplying in and harming a range of environments (Jnani and Ray, 2024). The virulence factors of *E. coli* O157:H7 are primarily attributed to a combination of specific factors that enable it to invade host tissues, evade immune responses, and inflict damage on the human body (Reiland *et al.*, 2014; Kademane and Dixit, 2023).

The major virulence factors of *E. coli* O157:H7 are shiga toxin, Intimin (*eae* gene), type III secreation system, lipopolysaccharides (endotoxins), hemolysins, flagella (H7 antigen), proteases, translocated intimin receptor (Tir) and plasmid-encoded virulence factors (pO157) (Lim *et al.*, 2010; Mainil, 2012). In addition to this there are also autotransporters (like serine protease autotransporter, and EspP), long polar fimbriae, EHEC hemorragic colitis pilli, IrgA homologue adhesin (Iha), ToxB, EHEC sereated proteins (Esp), acid resistance system, cytolethal distending toxin (CDT), heat-stable enterotoxin 1 (Stx1), and iron acquisition systems (Law, 2000; Kolodziejek *et al.*, 2022).

One of the most critical virulence factors of *E. coli* O157:H7 is Shiga toxin (Stx1 and Stx2). This potent cytotoxin disrupts protein synthesis in host cells, leading to cell death and significant gastrointestinal symptoms, including severe diarrhea, abdominal cramps, and vomiting (Obrig, 1997). Stx can enter the bloodstream, posing a risk for systemic complications such as HUS, a condition characterized by kidney failure and potentially life-threatening outcomes (Melton-Celsa, 2014). Shiga toxin also mediates local damage in the colon, which results in bloody diarrhoea, haemorrhagic colitis, necrosis and intestinal perforation (Kaper *et al.*, 2004).

Adhesion factors also play a crucial role in the pathogenicity of *E. coli* O157:H7. The strain is equipped with fimbriae and various surface proteins that facilitate adhesion to the intestinal epithelium (LeBlanc, 2003). This ability to cling to the intestinal lining is essential for

colonization, allowing the bacteria to resist flushing from the gastrointestinal tract during peristalsis. Once attached, *E. coli* O157:H7 can establish a niche in the gut, promoting further invasion and facilitating the production of Shiga toxin (Reiland *et al.*, 2014).

The Type III secretion system (T3SS) is another significant virulence factor that enhances the pathogenic potential of *E. coli* O157:H7 (Sharma *et al.,* 2012). This sophisticated molecular syringe-like apparatus allows the bacteria to inject effector proteins directly into host cells. These effector proteins manipulate host cell signaling pathways, promoting bacterial survival while simultaneously disrupting normal cellular functions. By modulating the host's immune response, *E. coli* O157:H7 can evade detection and destruction, further establishing its presence within the host (Wang *et al.*, 2024b).

Additionally, *E. coli* O157:H7 produces lipopolysaccharides (LPS), components of the outer membrane that can trigger intense inflammatory responses in the host. The lipopolysaccharide of *E. coli* O157:H7 is considered to be an important virulence factor; it is a mediator of the inflammatory response and synergy has been noted between LPS and the verotoxin (Louise and Obrig, 1992). It is a potential receptor for bacteriophage and a likely candidate for inducing protective immunity (Currie, 1999). While the immune response is crucial for controlling infections, the overwhelming inflammation induced by LPS can lead to tissue damage and contribute to the symptoms of infection (Reiland *et al.*, 2014; Amemiya *et al.*, 2023).

2.6. Pathogenesis of *Escherichia coli* O157:H7

Pathogenicity of *Escherichia coli* O157:H7 is encoded by a variety of plasmid, bacteriophage and chromosomal genes (Kiranmayi *et al.,* 2010). The pathogenesis of *E. coli* O157:H7 involves several key factors and mechanisms. The first one was adherence and colonization step. *E. coli* O157:H7 possesses adhesins, such as intimin (encoded by the *eae* gene), which facilitate its adherence to the intestinal epithelium (Frankel *et al*., 1998; Kenny and Jepson, 2000).

After adherence and colonization, type III secretion system (T3SS) starts to inject effector proteins into host cells, which manipulate host cell signaling pathways and cytoskeletal rearrangements. This system is encoded by the locus of enterocyte effacement (LEE) Pathogenicity Island (Clarke *et al.*, 2003; Kaper *et al.*, 2004). Then attaching and effacing lesions are induced on the intestinal epithelium due to the injected effector proteins. Attaching and effacing lesions are characterized by the intimate attachment of bacteria to the host cell membrane and localized destruction of microvilli (McDaniel, 1995; Nataro and Kaper, 1998).

Shiga toxins, potent cytotoxins produced by *E. coli* O157:H7, inhibit protein synthesis in host cells by removing a specific adenine residue from the 28S ribosomal ribonucleic acid of the 60S subunit of the ribosome, leading to cell death (Brigotti, 2014). The inflammatory response triggered by infection with *E. coli* O157:H7 in the intestinal mucosa lead to the recruitment of immune cells, release of pro-inflammatory cytokines, and disruption of the intestinal barrier function. Excessive inflammation contributes to the clinical manifestations of infection, including diarrhea and hemorrhagic colitis (Melton-Celsa, 2014; Pearson and Hartland, 2015).

2.7. Public Health Importance *Escherichia coli* O157:H7

Escherichia coli O157:H7 has a significant threat to public health worldwide and is is responsible for 20% of foodborne outbreaks worldwide (Pal and Ayele, 2017). Infection with *E. coli* O157:H7 in human presents with a wide spectrum of clinical manifestations including asymptomatic carriage, non bloody diarrhea, hemorrhagic colitis, the hemolytic-uremic syndrome, and thrombotic thrombocytopenic purpura (Duffy *et al.*, 2006; Scallan *et al.*, 2011; Sharma *et al.*, 2012). Additionally, individuals may experience nausea, vomiting, and sometimes even fever, though fever is less common. Not only is *E. coli* O157:H7 an important agent for hemorrhagic colitis, it is also one of the leading causes of bacterial diarrhea (Su and Brandt, 1995; Yun *et al.*, 2023).

The incubation period of *E. coli* O157:H7 infection can range from 3 to 8 days. Most patients recover within 10 days but in vulnerable patients, particularly young children and the elderly,

the infection can progress to more severe complications like HUS, which involves kidney failure, thrombocytopenia, and hemolytic anemia (Tarr *et al.*, 2005; Ho *et al.*, 2013; Yun *et al.*, 2023). The clinical progression of *E. coli* O157:H7 infection in children has been well documented, and includes a 3-day incubation period, followed by bloody diarrhea and HUS in about 15% of patients (Su and Brandt, 1995).

2.8. Control Strategies of *Escherichia coli* O157:H7

Effective control of *E. coli* O157:H7 requires a multi-faced approach. In addition to reducing the frequency and intensity of *E. coli* O157:H7 fecal shedding by cattle exposed to the organism, control of *E. coli* O157:H7 in environmental sources such as water troughs, feed and manure is critical in breaking the cycle of infection and re-infection of livestock (Betts, 2000). Foods of animal origin such as milk and dairy products and meat may be contaminated with *Escherichia coli* O157:H7 during production and processing, and the pathogen may survive or grow during processing operations, highlighting the need for well-designed and validated hazard analysis critical control point (HACCP) management systems (Duffy and McCabe, 2014).

The survival and growth of *E. coli* O157:H7 in foods are dependent on the interactions of numerous factors, including temperature, pH and water availability. The *E. coli* O157:H7 strain is very hardy and can survive for extended periods in water and soil, in dry conditions, and under frozen and refrigerated temperatures. The strain can also adapt to acidic conditions (Betts, 2000). It can be destroyed by thorough cooking or pasteurization. Consumers should always practice safe food handling and preparation measures. Root crops and leafy vegetables have the greatest risk of infection from manure application to soil. They can also become contaminated through direct or indirect contact with infected cattle, deer, and sheep (Duffy and McCabe, 2014).

Controlling *Escherichia coli* O157:H7 involves implementing strict hygiene protocols in animal husbandry to reduce fecal shedding of the bacteria and employing sanitation practices in processing facilities to prevent cross-contamination of food products. Additionally,

monitoring and testing programs are often put in place to detect and mitigate potential sources of contamination before they reach consumers (Jangid *et al.*, 2024)

Prompt diagnosis and treatment of infections are essential to prevent and control *E. coli* O157:H7 infection. Public health interventions are equally important in controlling the spread of *E. coli* O157:H7 within communities and the environment (Phillips, 1999). Surveillance systems are established to monitor for outbreaks and identify potential sources of contamination, allowing for rapid response and implementation of control measures. Educational campaigns are often deployed to raise awareness about the risks associated with *E. coli* O157:H7 and promote preventive measures such as safe food handling practices and proper hand hygiene (Brabban *et al.*, 2004).

2.9. Treatment of *Escherichia coli* O157:H7 Infection

There's no specific treatment for *E. coli* O157:H7 infection (Su and Brandt, 1995; Holtz *et al*., 2009**)**. Treatment focuses on managing symptoms and complications such as anemia and renal failure. Antibiotics haven't been proven to shorten the illness, and research on individual antibiotics is limited. Some studies suggest that antibiotics might not affect the duration of the illness (Remis *et al*., 1984; Ostroff *et al.*, 1989). Some studies even suggest that antibiotics might increase the risk of serious complications like hemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) or prolong diarrhea (Smith *et al.*, 2012).

In vitro studies suggest that most *E. coli* O157:H7 strains are susceptible to antibiotics. However, some antibiotics, especially at low doses, might actually increase the release of Shiga-toxins that can lead to serious health problems like HUS (Panos *et al*., 2006).The goal of treating *E. coli* O157:H7 is to remove the bacteria from the intestine without causing it to start producing toxins. Recent treatment approaches have included using antisera or monoclonal antibodies (Mühlen and Dersch, 2020).

2.10. Antimicrobial Susceptibility Pattern of *Escherichia coli* O157:H7

Antimicrobial resistance (AMR) is widely acknowledged as a major global health threat affecting both human and animal populations. This issue crosses international boundaries, impacting countries regardless of healthcare advancements or economic status, posing risks for both developed and developing nations (Queenan *et al*., 2016). Similarly, antimicrobial susceptibility pattern of *Escherichia coli* O157:H7 is an important concern due to its potential impact on human and animal health. This pathogen's susceptibility to specific antibiotics varies significantly, reflecting differences in regional practices, antibiotic use, and environmental factors (Abreham *et al.,* 2019). Recent studies highlight notable findings regarding its susceptibility and resistance profiles, with implications for antibiotic stewardship and public health.

The mechanisms of antimicrobial resistance in *E. coli* O157:H7 is the acquisition of resistance genes through horizontal gene transfer. These genes encode proteins that confer resistance to specific antibiotics by various mechanisms, such as enzymatic degradation, modification of drug targets, or efflux of the antibiotic from the bacterial cell (Reygaert, 2017). The presence of mobile genetic elements, such as plasmids and transposons, facilitates the spread of resistance genes within bacterial populations, leading to the emergence of multidrug-resistant strains (Javadi *et al.,* 2017; Ullah and Ali, 2021).

3. MATERIALS AND METHODS

3.1. Study Area Description

The study was conducted in Hawassa town, including Lake Hawassa, and Yirgalem town. Hawassa town is an administrative capital of the Sidama regional state and located in 275 km south of Addis Ababa. Hawassa town has a total area of about 50 km^2 and situated at an altitude of 1700 to 1750 m above sea level and lies within the geographic coordinates between 06°83' to 7°17' N and 38°24' to 38°72' E. The mean annual range of precipitation and temperature are 950-1200 mm and 20°C to 27°C, respectively (Gedecho, 2015).

Lake Hawassa is the smallest among the rift valley lakes of Ethiopia and located between 06°58′ to 07° 14′ North latitudes and 38° 22′ to 38° 28′ East longitudes with an altitude of 1697 m above sea level. The lake is a shallow lake with average depth of 13.3 m and maximum depth of 23.4 m. It has a surface area of 90 km^2 and a drainage area of 1250 km^2 and a water storage capacity of the lake is 1.36 km^3 (Tilahun and Ahlgren, 2010). The common landing site and fish market of Lake Hawassa fishery are Gudumalle or Amoragedel (Tekle-giorgis *et al.*, 2017) and at other shores of the lake. The lake is fed by Tikur Wuha River, a perennial river that drains water from eastern escarpment and a swampy area of the former Lake Shallo (Chaleleka) (Makin *et al.*, 1975).

Lake Hawassa plays an important role in the livelihoods of local people, serving as a source of income from fishing, drinking water, irrigation, and tourism (NRSP, 2023; Tibebe *et al.*, 2023). Yilma *et al*. (2020) reported that fish from Lake Hawassa are a valuable nutritional resource for the local community, providing a rich source of protein and essential fatty acids. The lake has a potential for 600 metric tons of fish production per year, where Nile tilapia (*Oreochromis Niloticus*) is the most popular fish species harvested in larger number followed by Catfish (*Clarias gariepinus*) and Barbus (*Labeobarbus intermedius*) (Patnaik, 2014; Tekle-giorgis *et al.*, 2017).

Yirgalem town is small town and administrative head of Dale woreda/district in Sidama regional state. It is located at $6^0$44'to $6^0$46'N latitude and $38^0$24' to $38^0$26'E longitude. The study area has an elevation of 1600 to 1960 meters. It is located 47 km from Hawassa and 311 km south of Addis Ababa, the capital of the country (Tekle-yohannes, 2019). Yirgalem Town has a moderate climate with a minimum and maximum annual temperature of 14 and 30°C, respectively. The study area obtained a bimodal rainfall with peaks in April, June, and August, with an annual rainfall of 1138–1690 mm (Yusuf *et al.*, 2018).

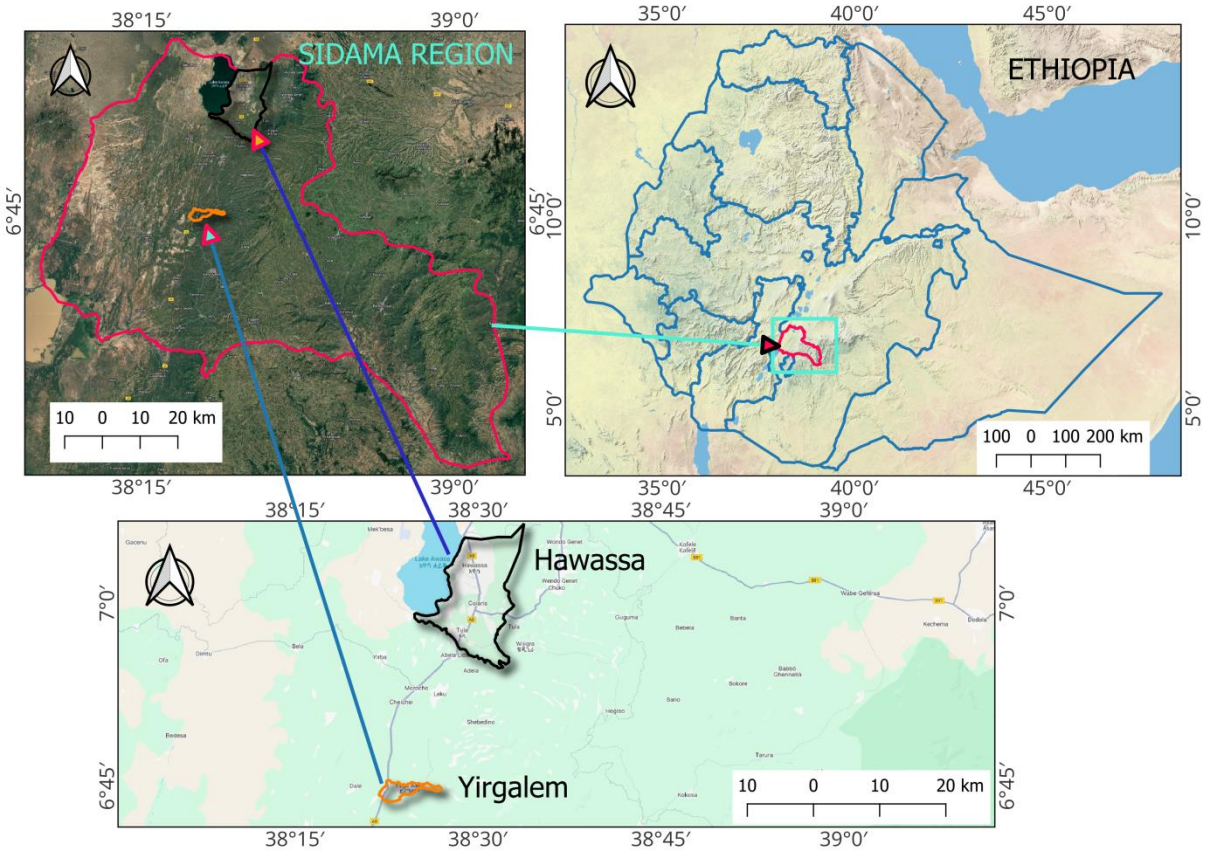


Figure 1: Map of the study area

3.2. Study Design and Sampling method

A cross-sectional study was conducted from December 2022 to July 2023 in Hawassa and Yirgalem towns, Sidama, Ethiopia, to isolate and identify *E. coli* O157:H7 from animal-origin foods and fish/fish landing sites. A total of 298 samples were collected from Hawassa and

Yirgalem towns of Sidama region, Ethiopia, they were selected purposively chosen based on factors such as logistics, the number of dairy farms, abattoirs, butcher shops, and fishery activities, as well as the high tourist traffic to Hawassa town and Lake Hawassa.

Four sub-cities in Hawassa (Addis Ketema, Mehal Ketema, Meneharia, and Tabor) and two in Yirgalem (Filwuha and Arada) were purposively selected based on their high number of butcher shops, dairy farms, and milk retailers, as identified from data provided by the Livestock and Fishery Development and Trade and Market Development sector offices of each municipality. Within these selected sub-cities, a simple random sampling approach was employed to select specific butcher shops, dairy farms, milk retailers, and from Lake Hawassa, fish samples harvested by local fishermen for inclusion in the study. Hawassa Municipality abattoir and Hawassa University abattoirs were purposively selected based on their convenience to collect samples of abattoir during their overnight operation.

3.3. Sample Collection and Transportation

Meat swab samples were collected from butcher shops and abattoirs following the procedures recommended by Arthur *et al*. (2004). Briefly, cotton-tipped swabs soaked in sterile buffered peptone water (HiMedia, India) were rolled across specific carcass areas (navel, plate, brisket, and fore shank). Swabs from the same carcass were pooled and placed in falcon tubes containing sterile buffered peptone water. Swabs were also taken from equipment such as cutting boards, carcass hangers, knives, and knife sharpeners using similar sterile procedures. These equipment swabs from each shop or abattoir were pooled in collection tubes containing 10 mL of sterile buffered peptone water. Hand swabs from workers were also collected and pooled in the same manner.

Milk samples were collected from dairy farms and milk vendors (shops selling milk and dairy products). Approximately 10 mL of milk was collected from each farm or vendor into sterile containers with 90 mL of sterile buffered peptone water (1:9 ratios). Swab samples were also taken from the milking environment, including milk collection and milking containers, as well as measuring plastics. These swabs were pooled and placed in sterile tubes with 10 mL of

sterile buffered peptone water. Hand swabs were also collected from workers who consented and pooled similarly.

Swab samples were collected from purchased fish at different fish landing sites around Lake Hawassa. Samples were taken from the fish skin (before processing) and fish muscle (after aseptic processing) and placed in falcon tubes containing 10 mL of sterile buffered peptone water. All samples were collected aseptically, with gloves used during handling and measures taken to avoid cross-contamination. Samples were transported in a cool box with ice packs to Hawassa University's Veterinary Microbiology Laboratory, where bacterial culturing and biochemical identification were performed.

3.4. Isolation and Biochemical Identification of *Escherichia coli* O157:H7

Upon arrival at the laboratory, all swab samples were incubated overnight at 37°C as a pre-enrichment step. For milk samples, approximately 10 mL of the sample was mixed with 90mL of buffered peptone water, followed by incubation at 37°C for 24 hours. After this incubation period, the samples were thoroughly homogenized, and 1 mL of the aliquot was transferred into 9 mL of Tryptone Soya Broth (HiMedia, India) in a 1:9 ratio for enrichment and incubated at 37°C for another 24 hours.

Following enrichment, the samples were inoculated onto MacConkey Agar (HiMedia, India), a selective and differential medium for *E. coli* growth, and incubated for 24 hours at 37°C. Presumptive *E. coli* colonies, identified as pink, round colonies, were retrieved and sub-cultured onto fresh MacConkey Agar for another 24 hours at 37°C to obtain isolated, distinctive colonies.

Colonies that appeared pure pink to red and round were further analyzed using the catalase and Gram stain tests. Colonies that were catalase-positive and Gram-negative were subjected to biochemical tests, including SIM (Sulfide, Indole, and Motility), TSI (Triple Sugar Iron), and Simmons citrate tests, following the guidelines of Quinn *et al.* (2002). The isolates that

were negative for sulfide production, positive for indole and motility tests, negative for the Simmons citrate test, and those with yellow butt and yellow slant and with gas production and no hydrogen sulfide production in TSI test were identified as *E. coli*.

A suspected *E. coli* isolates were then inoculated onto nutrient agar and incubated at 37°C for 24 hours to obtain pure *E. coli* colonies. To further differentiate *E. coli* O157:H7, *E. coli* isolates were inoculated onto sorbitol MacConkey Agar, a differential medium designed to distinguish *E. coli* O157:H7 from other strains. After 24 hours of incubation at 37°C, *E. coli* O157:H7 appeared as colorless colonies due to its inability to ferment sorbitol, while other *E. coli* strains appeared pink as they ferment sorbitol (Soomro *et al.*, 2002). All presumptive *E. coli* O157:H7 isolates were preserved at -20°C in brain heart infusion broth with 40 µL of 20% glycerol in 60:40 ratios in 2 mL cryovials until PCR identification.

3.5. PCR Detection of *Escherichia coli* O157:H7

E. coli O157:H7 genomic DNA was isolated from fresh cultures by the boiling method as described earlier (Lesiani *et al.*, 2023). Briefly, about 5 µL of homogenized presumptive *E. coli* O157:H7 isolate suspension grown overnight at 37^{o}C in brain heart infusion broth culture was added to 100 µL of sterilized DNAse free water. To obtain a DNA lysate, the suspension was vortexed for homogenization of cells and boiled at 100^{o}C for 10 minutes using a thermocycler PCR machine (Esco Scientific) and cooled to 4^{o}C until the next step.

All presumptive *E. coli* O157:H7 isolates were subjected to multiplex PCR analysis using the forward and reverse primers listed in Table 1. The primers were designed to target specific genes: *rfb*O157 (encoding the cell wall lipopolysaccharide antigen O157), *fli*CH7 (encoding the flagellar antigen H7), and Stx2 (encoding the Shiga- toxin 2).

Table 1: Primers (forward and reverse primers) setup

Primer	**Target gene**	**Primer sequence**	**Gene product**	**Product size (bp)**
PF8	*rfb*O157	5'CGTGATGATGTTGAGTTG3'	LPS O157	420
PR8		5'AGATTGGTTGGCATTACTG3'		
FLICH7-F	*fliCh7*	5'GCGCTGTCGAGTTCTATCGAGC3'	H7	625
FLICH7-R		5'CAACGGTGACTTTATCGCCATTCC3'		
SLTII-F	*Stx*2	5'GTTTTTCTTCGGTATCCTATTCC3'	Stx2	484
SLTII-R		5'GATGCATCTCTGGTCATTGTATTAC3'		

Note*: fliCh7*→ encodes for *E. coli* structural flagellar antigen H7; *rfb*O157→ encodes for the *E. coli* somatic antigen O157; *Stx*2→encodes the shiga toxin 2 (extracellularly secreted)

For the multiplex PCR analysis, a final of 25 µL volume of PCR reaction mix was made by mixing 2.5 µL of the 10X reaction buffer, 0.5 µL of deoxyribonuclease triphosphates (dNTPs), 0.5 µL of each of the forward and reverse primers, 0.05 µL TaqPolymerase, and 19.95 µL of DNase/RNase free water and 1µL template DNA. The multiplex PCR amplification was performed by thermal cycler (Esco Scientific) with the cycling conditions described by Fratamico *et al.* (2000) with slight modification. Briefly, initial denaturation was made at 95^{o}C for 3 minutes, 34 cycles of denaturation at 95^{o}C for 20 seconds, annealing at 54^{o}C for 1 minute and extension at 72^{o}C for 1 minute. The final extension was done at 72^{o}C for 7 minutes. The PCR products were then held at 4^{o}C prior to gel electrophoresis.

The PCR products were resolved in 1.5% (w/v) agarose gel stained with 3 µL of Ethidium bromide in 1x TBE buffer for 1:00 hour. A 1 kb DNA ladder was used as a standard molecular size marker. The bands were then visualized by UV transillumination (BTS-20) and photographed.

3.6. Antimicrobial Susceptibility Testing

The antibiotic susceptibility test was performed on all PCR-confirmed *E. coli* O157:H7 isolates using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (Kirby *et al.*, 1966). Each confirmed isolate was tested for susceptibility to a panel of 11 antibiotics commonly used in both animals and humans. The antibiotics and their respective disc potencies are detailed in Table 2. In summary, a pure, fresh colony of *E. coli* O157:H7 was inoculated into nutrient broth and incubated at 37°C for 5–6 hours, with the culture tube being periodically checked for bacterial growth.

Then the turbidity of the growth was adjusted using 0.85% saline solution until it matched with a 0.5 McFarland turbidity standards. Then sterile cotton tipped swab was soaked into the emulsified broth and the bacteria were swabbed evenly over the surface of Muller Hinton agar plate and the plates were kept at room temperature for 15 minutes to dry.

Each antibiotic disc was carefully placed on the agar plates and left on the bench (in front of a Bunsen burner) for 15 minutes to allow the discs to adhere to the media. The plates were then incubated for 18 to 24 hours at 37°C. After incubation, the results were measured using a digital caliper, recorded, and classified as resistant, intermediate, or susceptible, following the guidelines and standards of the CLSI (2020) manual (Table 2).

Table 2: Antimicrobials used and inhibition zone interpretation for Enterobacteriaceae

Drug class	Name of drugs	Code	Dose. in μg	Inhibitory zone diameter breakpoints, nearest whole mm		
				Susceptible	Intermediate	Resistance
Fluoroquinolones	Ciprofloxacin	CIP	5	≥18	13-17	≤12
	Nalidixic acid	NA	30	≥19	14-18	≤13
Phenicol	Chloramphenicol	C	30	≥18	13-17	≤12
Cephalosporins	Cephalothin	KF	30	≥18	15-17	≤14
	Ceftazidime	CAZ	30	≥21	18-20	≤17
	Ceftriaxone	CTR	30	≥23	20-22	≤19
Tetracycline	Tetracycline	OT	30	≥15	12-14	≤11
Penicillin	Ampicillin	AMP	10	≥17	14-16	≤13
Aminoglycosides	Streptomycin	S	10	≥15	12-14	≤11
	Gentamycin	CN	10	≥15	13-14	≤12
Lincosamide	Clindamycin	CD	2	≥21	15-20	≤14

Isolates exhibiting multiantibiotic resistance were also recorded. Isolates were considered as multidrug resistant (MDR) when they are found resistant against three or more antibiotic classes (Magiorakos *et al.*, 2012) The multidrug resistance index (MDRI) was calculated to assess the extent of antibiotic resistance among the tested isolates. The MDRI was determined according to Krumperman's (1983) formula:

$$MDRI = \frac{Number\ of\ antibiotics\ to\ which\ the\ isolates\ were\ resistant}{Total\ number\ of\ antibiotics\ tested}$$

Where: MDRI= Multidrug resistance index

Moreover, *E. coli* O157:H7 isolates were also phenotypically examined for the production of extended spectrum beta-lactamase (ESBL) enzyme. ESBL enzymes are plasmid-encoded enzymes that produced by some bacteria, including *E. coli* strains, that confer resistance to a

broad range of beta-lactam antibiotics, particularly to third-generation cephalosporins like ceftazidime, ceftriaxone, and cefotaxime (Rawat and Nair, 2010).

In this study, the phenotypical ESBL screening test procedure was done by using two cephalosporin drugs, ceftazidime and ceftriaxone. The disc diffusion method of the antimicrobial susceptibility test was made on Muller-Hington agar to screen ESBL bacterial isolates by measuring the clear zone of inhibition for the ceftazidime drug and ceftriaxone. If the zone of inhibition was ≤ 22 mm for ceftazidime or ≤ 25 mm for ceftriaxone, the isolate was suspected as a possible ESBL producer (Giske *et al*., 2013; CLSI, 2020).

To confirm this, the suspected isolates were retested with ceftazidime and ceftriaxone drugs with their respective combination of clavulanic acid in double disc diffusion method. If the zone of inhibition for their combination of clavulanic acid was at least 5 mm larger than the zone of inhibition for ceftazidime and ceftriaxone alone, the isolate was confirmed as an ESBL producer according to CLSI (2020) guidelines.

3.7. Data Management and Statistical Analysis

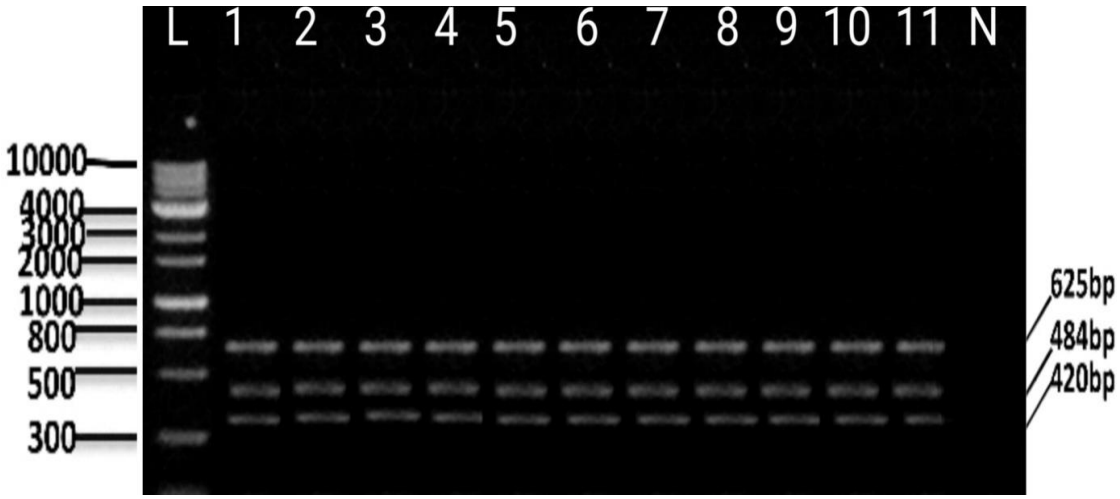
All the data obtained at different steps of this study were entered into Microsoft Excel® 2010 spreadsheet, filtered and coded for further statistical analysis by SPSS Version 20. Descriptive statistics were used to determine percentages and fisher's exact test was employed to compare percentages for different variables. Differences were recorded as statistically significant only when the P-value is < 0.05.

3.8. Ethical Considerations

This study received ethical approval from the Research Ethics Review Committee of Hawassa University, College of Natural and Computational Sciences (Ref. No. RERC/009/22). Additionally, support letters were obtained from the Sidama Regional State, as well as from Hawassa town and Yirgalem town, to ensure smooth implementation of the study.

4. RESULTS

In this study, a total of 298 samples were analyzed, of which 11 (3.7%) tested positive for *E. coli* O157:H7 contamination. Among the sample types, fish exhibited the highest contamination rate at 6.25% (4 out of 64), followed by beef at 5.6% (7 out of 125), while no contamination was found in milk samples (0 out of 109). The difference in *E. coli* O157:H7 occurrence among these sample types was statistically significant (p = 0.013). The amount of *E. coli* O157:H7 occurrence in Yirgalem (6.1%) was greater than Hawassa (3.2%) but there was no statistically significant difference in between locations (p = 0.398).



Note: L: 1 kbDNA marker; Lane 1-11: Positive samples; N: Negative control (water); 625bp =*fliCh7*; 484bp = *stx2*; 420bp =*rfbO157*

Figure 2: Representative gel image of PCR analysis

Table 3: *E. coli* O157:H7 occurrence in different animal origin food types and study area

Variables	Sample size	*E. coli* O157:H7		Fisher's exact test P value
		Count	Percent (%)	
Beef	125	7	5.6	0.013
Milk	109	-	-	
Fish	64	4	6.25	
Total	298	7	3.7	
Hawassa	249	8	3.2	0.398
Yirgalem	49	3	6.1	
Total	298	7	3.7	

Additionally, beef samples from Yirgalem had a higher contamination rate (18.8%, 3 out of 16) compared to those from Hawassa, where only 3.7% (4 out of 109) of *E. coli* O157:H7 was found in beef and related environmental samples. This difference was also statistically significant ($p = 0.044$). Furthermore, beef samples from butcher shops showed a higher contamination rate (7.6%, 5 out of 66) compared to those from abattoirs (3.4%, 2 out of 59), which was not statistically significant as well ($p = 0.445$).

Table 4: *E. coli* O157:H7 occurrence in beef in terms of Study area and sample source

Beef sample	Variables	Sample size	*E. coli* O157:H7		Fisher's exact test P value
			Count	Percent (%)	
Study area	Hawassa	109	4	3.7	0.044
	Yirgalem	16	3	18.8	
	Total	125	7	5.6	
Sample sources	Abattoir	59	2	3.4	0.445
	Butchers shop	66	5	7.6	
	Total	125	7	5.6	

The samples from beef meat origin showed a varying occurrence rates of *E. coli* O157:H7, 5.6% from a meat swab, 9.5% from an environmental swab, and 0% from a hand swab, still it

was not statistically significant difference (p = 0.674) (Table 5). From abattoir origin samples, *E. coli* O157:H7 positive isolates were originated only from meat swabs while it was from meat swab (60%) and environmental surface swab specimens (40%) from butcher shops.

Table 5: *E. coli* O157:H7 occurrence in beef in terms of beef specimen types

Beef specimen types	Sample size	*E. coli* O157:H7		Fisher's exact test P value
		Count	Percent (%)	
Meat swab	90	5	5.5	0.674
Environmental swab	21	2	9.5	
Hand swab	14	-	-	
Sub total	125	7	5.6	

Of the 64 total fish samples examined, 6.25% (4 out of 64) were found to be contaminated with *E. coli* O157:H7, regardless of the collection sites. A notably higher percentage (75%) of these isolates were recovered from *O. niloticus* (Nile tilapia), while only one isolate (25%) was detected in samples from *C. gariepinus* (African catfish).

In terms of collection sites, fish samples from Gudumalle had a higher contamination rate (7.3%, 3 out of 41) compared to samples from other sites, where only one sample (4.3%, 1 out of 23) was contaminated. Most of the *E. coli* O157:H7 isolates (8.6%, 3 out of 35) were found in fish skin swabs, whereas only one isolate (3.4%, 1 out of 29) was detected in fish muscle swabs. However, all observed differences in contamination rates were not statistically significant (P > 0.05) (Table 6).

Table 6: Occurrence of *E. coli* O157:H7 in fish species, specimens and fish landing sites

	Variables	Sample size	*E. coli* O157:H7		Fisher's exact test P value
			Count	Percent (%)	
Fish species	*O. niloticus*	37	3	8.1	0.632
	C. gariepinus	27	1	3.7	
Fish specimen	Muscle swab	29	1	3.4	0.620
	Skin swab	35	3	8.6	
Fish landing sites	Gudumalle	41	3	7.3	1.000
	Other sites	23	1	4.3	
	Total	64	4	6.2	

Antibiotic susceptibility testing was performed on all 11 PCR-confirmed *E. coli* O157:H7 isolates using a panel of 11 antibiotics from different antimicrobial classes. The results showed that all *E. coli* isolates were 100% susceptible to ciprofloxacin (CIP 5µg), nalidixic acid (NA 30µg), chloramphenicol (C 30µg), cephalothin (KF 30µg), ceftazidime (CAZ 30µg), ceftriaxone (CTR 30µg), tetracycline (T 30µg), and gentamicin (CN 10µg). However, 81.8% (9 out of 11) of the isolates were resistant to ampicillin (AMP 10µg), and 45.5% (5 out of 11) showed resistance to streptomycin (S 10µg). Furthermore, all *E. coli* O157:H7 isolates were 100% resistant to clindamycin (CD 2µg) (Table 7).

Table 7: Antibiotic susceptibility profile of *E. coli* O157:H7 isolates against 11 antibiotics

Antibiotic discs	Code of discs	Susceptible N (%)	Intermediate N (%)	Resistance N (%)
Ciprofloxacin	CIP5	11 (100%)	-	-
Nalidixic acid	NA30	11 (100%)	-	-
Chloramphenicol	C30	11 (100%)	-	-
Cephalothin	KF30	11 (100%)	-	-
Ceftazidime	CAZ30	11 (100%)	-	-
Ceftriaxone	CTR30	11 (100%)	-	-
Tetracycline	T30	11 (100%)	-	-
Ampicillin	AMP10	-	2 (18.2%)	9 (81.8%)
Streptomycin	S10	-	6 (54.5%)	5 (45.5%)
Gentamycin	CN10	11 (100%)	-	-
Clindamycin	CD2	-	-	11 (100%)

Multidrug resistance was observed in 45.45% (5 out of the 11) of PCR confirmed isolates, with only one MDR pattern to three drugs, clindamycin, ampicillin, and streptomycin. The calculated multidrug resistance index was 0.273.Moreover, screening for extended-spectrum beta-lactamase (ESBL) production, performed using ceftazidime (CAZ30) and ceftriaxone (CTR30) discs, showed no positive results for ESBL production. The minimum observed inhibition zones were 24 mm for ceftazidime and 29 mm for ceftriaxone, both exceeding their respective ESBL screening breakpoints of 22 mm and 25 mm, indicating the absence of ESBL producers.

5. DISCUSSION

Pathogenic *Escherichia coli* is significant water- and foodborne pathogen, associated with a wide range of gastrointestinal and systemic symptoms, from mild to severe. Infections caused by pathogenic strains like *E. coli O157*:H7 pose a major public health challenge globally, particularly when found in animal-derived foods, which are a significant risk factor for transmission (Gambushe *et al.*, 2022). This study aimed to assess the occurrence and antimicrobial susceptibility of *E. coli* O157:H7 in various animal-origin foods, including beef, milk, and fish, collected from Hawassa and Yirgalem towns, Sidama, Ethiopia.

The overall occurrence of *E. coli O157*:H7 in this study was 3.7%, which aligns with a 3.5% prevalence reported in Bishoftu (Bedasa *et al.*, 2018) and a 4% overall prevalence found in a meta-analysis by Assefa (2019). However, this finding is relatively lower than some other studies conducted both in Ethiopia and internationally. For example, other Ethiopian studies have reported higher prevalence rates: 6.5% by Abebe *et al.* (2023) in Dessie and Kombolcha, 14.3% by Gugsa *et al.* (2022) in Mekele city, and 10.4% by Abebe *et al.* (2014) in selected districts of the Tigray region. Internationally, Islam *et al.* (2010) reported a significantly higher prevalence of 52.4% from meat, milk, and juice samples in Bangladesh. In contrast, the 3.7% occurrence observed in this study is higher than other reports, such as 2.1% in Ethiopia (Geresu and Regassa, 2021) and 1.5% in Nigeria (Ayodele *et al.*, 2020).

These discrepancies in prevalence could be attributed to several factors, including differences in methodology, like variations in isolation and identification methods. For instance, while most studies used only culture and biochemical methods for *E. coli* O157:H7 identification, Ayodele *et al.* (2020) employed molecular techniques like PCR for more accurate identification. In the current study, both biochemical and molecular methods, including PCR, were used to enhance the specificity and sensitivity of pathogen detection. PCR-based identification is widely recognized as a more reliable and accurate method for detecting pathogenic bacteria, such as *E. coli* O157:H7, compared to traditional culture, serological assays, and biochemical tests (Gambushe *et al.*, 2022; Hariri, 2022).

This study found that the occurrence rate of *E. coli* O157:H7 in beef samples was 5.6%, which is consistent with previous findings in Ethiopia, such as 4.97% (Engda *et al.*, 2020), 6.25% (Sekhar and Hirbaye, 2024), and 5.4% (Sebsibe and Asfaw, 2020), in Negele and Jimma towns, Ethiopia, respectively. However, this rate was higher than other Ethiopian studies, including 2.7% from Hawassa (Atinafae *et al.*, 2017), 4.3% from Central Ethiopia (Abunna *et al.*, 2023), 2.7% from Bedele (Fikadu *et al.*, 2023), and 4.0% from Dessie and Kombolcha (Abebe *et al.*, 2023). Similarly, international studies also reported lower rates, with 3.1% from Egypt (Ahmed and Shimamoto, 2014), 1.7% from Egypt (Ahmed *et al.*, 2017), and 0.2% from Nigeria (Mailafia *et al.*, 2017).

On the other hand, the 5.6% prevalence observed here was lower than other reports from Ethiopia and beyond, such as 12.0% from Addis Ababa (Hamid *et al.*, 2018), 8.87% from the Awi zone (Tarekegn *et al.*, 2023), 8.9% from Bahir Dar city (Ayenew *et al.*, 2021), and 9.1% from Ambo town (Tadese *et al.*, 2021). Internationally, rates as high as 35.1% in South Africa (Onyeka *et al.*, 2021), 60% in Nigeria (Lennox *et al.*, 2020), and 25.46% in India (Vijayan *et al.*, 2017) have also been reported.

The variations in these findings could be due to differences in study methodology, sampling techniques, isolation and detection methods, and geographical and environmental factors. Additionally, local standards, hygiene practices, and sanitation protocols in slaughterhouses and butcher shops may influence contamination rates, as could population density and other regional factors.

In this study, no *E. coli* O157:H7 was detected in hand swab samples from abattoir or butcher shop workers, consistent with other studies (Abdissa *et al.*, 2017; Atinafae *et al.*, 2017; Sebsibe and Asfaw, 2020; Amente *et al.*, 2022). However, the bacteria were found on knives, cutting boards, and meat hooks, which agrees with findings from other research (Abdissa *et al.*, 2017; Beyi *et al.*, 2017; Sebsibe and Asfaw, 2020; Abunna *et al.*, 2023; Fikadu *et al.*, 2023). This highlights the importance of environmental factors in beef contamination. Poor sanitation practices, contaminated surfaces, and inadequate cleaning protocols are likely

contributing to the high prevalence of *E. coli* O157:H7 in meat (Atinafae *et al.*, 2017; Abebe *et al.*, 2023).

On the other hand, the overall prevalence (6.25%) of *E. coli* O157:H7 recorded from fish samples in this study was significantly lower than the 14.1% prevalence reported by Walelign *et al.* (2022) in their analysis of fish from Lake Abbaya and the Gilgel Gibe-3 artificial electric dam. Several factors may explain these observed differences, including variations in sample size, sampling locations, isolation techniques, and geographic conditions.

Furthermore, methodological differences played a crucial role, as the earlier study relied solely on standard biochemical tests for *E. coli* O157:H7 identification. In contrast, the present study utilized more sensitive PCR analysis, targeting the *fliC*h7, *rfb*O157, and Stx2 genes which are highly conserved genetic markers among *E. coli* strains which could enhance the detection accuracy and specificity.

Several global studies have reported varying prevalence rates of *E. coli* O157:H7in fish, reflecting regional and environmental variability in contamination levels. For example, Onmaz *et al.* (2020) reported a lower prevalence of 1.4% in Turkey, whereas Ribeiro *et al.* (2016) found a comparable rate of 7.29% in Brazil. Other studies, such as Bedane *et al.* (2024) from lakes in Central Oromia, Ethiopia, and Thampuran *et al.* (2005) from fish sold at retail markets in Cochin, India, reported no detection of *E. coli* O157:H7. These disparities might be due to differences in local environmental factors, fish species, sampling methods, and regional food safety practices.

In contrast, other studies have reported low but detectable levels of *E. coli* O157:H7. For instance, Assefa *et al.* (2019) reported a prevalence of 1.46% in fish samples from Lake Hayq and the Tekeze Dam in Northern Ethiopia, while Tuyet *et al.* (2006) found a prevalence of 4.7% in Bangui and N'Goila of the Central African Republic. Similarly, Tilahun and Engdawork (2020) observed a 2.3% prevalence of *E. coli* O157:H7 in Lake Hawassa, a site that was also included in the current study. These findings lower than the overall prevalence in

the present research; underscore the variability in contamination rates, influenced by environmental conditions and geographical differences.

In this study, none of the milk samples were found to be contaminated with *E. coli* O157:H7, resulting in a 0% occurrence rate. This finding aligns with similar studies conducted in Ethiopia and internationally. For instance, Dadi *et al.* (2020) from Sebeta, Ethiopia, and Addo *et al.* (2011) from Ghana reported no detection of *E. coli* O157:H7 in raw milk samples. Similarly, Swai and Scotchman (2011) in Tanzania, Sancak *et al.* (2015) in Turkey, and Hancock *et al.* (1994) in Washington State, USA, also found a 0% prevalence of *E. coli* O157:H7 in milk samples.

The absence of contamination in this study could be attributed to several factors, including the type of milk sampled, the methodologies employed for sampling and processing, and the possible implementation of improved hygiene practices during milking and milk handling. Enhanced hygienic practices can significantly reduce bacterial contamination, particularly in areas where food safety standards have improved.

However, other studies have reported varying levels of *E. coli* O157:H7 contamination in milk. Some studies from Ethiopia, such as 16.7% in Dessie and Kombolcha (Abebe *et al.*, 2023), 12% in Bishoftu (Bedasa *et al.*, 2018), 8.9% in Assela (Abunna *et al*., 2018), 4.08% in Central Ethiopia (Dejene *et al.*, 2022), 2.9% in Assosa (Disassa *et al.*, 2017), and 2.37% in Modjo (Welde *et al*., 2020), indicates the presence of wide range variation in contamination rates. Globally, 27.08% in Iraq (Abbas *et al.*, 2012), 18.75% in Malaysia (Lye *et al*., 2013), and 11% in South Africa (Msolo *et al.,* 2016), documented higher levels of contamination. On the other hand, lower prevalence rates were recorded by Karns *et al.* (2007), 0.02% in the United States; Inat *et al.* (2017), 0.66% in Turkey; Amer and Solimen (2004), 1% in Egypt; and Ivbade *et al.* (2014), 2% in Nigeria.

These variations in contamination levels could be explained by differences in environmental conditions, milk handling practices, and sampling techniques. In regions with higher contamination rates, factors such as poor hygiene during milking, improper storage, or cross-

contamination could contribute to the presence of *E. coli O157*:H7. Conversely, strict adherence to food safety protocols can help minimize contamination risks, as seen in the present study. These findings underscore the importance of maintaining stringent hygiene standards in dairy production to ensure the microbiological safety of milk (Amer and Solimen, 2004; Meshref, 2013; Abunna *et al.*, 2018).

In this study, the antimicrobial susceptibility profiling of *E. coli* O157:H7 isolates revealed a high level of susceptibility to most of the antibiotics tested. Notably, all isolates were completely susceptible to several common antibiotics, including ciprofloxacin, nalidixic acid, chloramphenicol, cephalothin, ceftazidime, ceftriaxone, gentamycin, and tetracycline. These antibiotics demonstrated 100% effectiveness, with all isolates showing full susceptibility. This suggests that these antibiotics remain highly effective for treating infections caused by *E. coli* O157:H7 in Ethiopia. The observed effectiveness could potentially reflect the limited exposure of this pathogen to these antimicrobials in local food production environments.

Consistent with the findings of this study, Sarba *et al.* (2023) also reported 100% susceptibility of *E. coli* O157:H7 to ciprofloxacin, ceftriaxone, ceftazidime, and nalidixic acid. Similarly, Gudisa *et al.* (2022) documented complete susceptibility to tetracycline, chloramphenicol, and nalidixic acid. Comparable results were also observed by Mesele *et al.* (2023) for chloramphenicol, ciprofloxacin, gentamicin, tetracycline, and nalidixic acid; and by Shubisa *et al.* (2022) for ciprofloxacin, gentamicin, and ceftriaxone. Additionally, Koev *et al.* (2020) reported a susceptibility rate of 96.6% for cephalothin, while other studies, such as those by Mesele *et al.* (2023) and Çadırcı *et al.* (2017), observed 100% resistance, reflecting variability in susceptibility patterns across regions or contexts.

However, in this study, most *E. coli* O157:H7 isolates exhibited resistance to clindamycin, ampicillin, and streptomycin. This resistance pattern is consistent with previous studies by Mesele *et al.* (2023), Haile *et al.* (2022), Gudisa *et al.* (2022), Sarba *et al.* (2023), Ababu *et al.* (2020), Ayenew *et al.* (2021), El Seedy *et al.* (2019), and Amer *et al.* (2018). The high rates of resistance may be attributed to the inappropriate and excessive use of these antibiotics for both therapeutic and prophylactic purposes in human and animal infections. Additionally, the

injudicious use of these and other antibiotics in animal production systems likely contributes to the development of resistance (Tilahun and Engdawork, 2020; Azabo *et al.*, 2022).

In this study, multidrug resistance (MDR) was observed in 45.45% (5 out of 11) of PCR confirmed *E. coli* O157:H7 isolates for three drug classes, resulting in a multidrug resistance index of 0.273 (27.3%). This finding was comparable with 51.8% MDR reported by Sebsibe and Asfaw (2020) but it is considerably lower than the 100% reported by Beyi*et al.* (2017), underscoring the importance of continued surveillance and judicious antibiotic use to prevent the escalation of resistance.

Importantly, no extended-spectrum beta-lactamase (ESBL)-producing *E. coli O15*7:H7 strains were detected, which is encouraging for antibiotic resistance management. ESBL production confers resistance to a broad range of beta-lactam antibiotics, complicating treatment options. However, the absence of ESBL producers in this study should not lead to complacency; ongoing monitoring is essential to detect emerging resistance patterns. As demonstrated by Ayalneh *et al.* (2024), who reported 71.1% ESBL production in *E. coli* O157:H7 strains from clinical samples, the potential for resistance remains a significant concern.

The limitations of this study includes the relatively small sample size for environmental and hand swab samples collected from beef and milk sources, with no environmental or hand swab samples obtained from fish processing sites and their surroundings. Additionally, the total sample size of 298 was limited and could have been increased to provide more robust findings. Furthermore, the study was geographically confined to two towns, which may have limited the generalizability of the results. The absence of a previously confirmed and preserved *E. coli* O157:H7 strain as a positive control during molecular detection was also a constraint, potentially affecting the accuracy of molecular analyses.

6. CONCLUSION AND RECOMMENDATIONS

This study aimed to investigate the contamination of animal-origin foods, including beef, milk, and fish, with *Escherichia coli* O157:H7 in Hawassa and Yirgalem towns, Sidama, Ethiopia. The findings revealed an overall occurrence rate of 3.7%, with the highest contamination detected in fish samples, followed by beef, while no contamination was found in milk samples, and the association between food types and level of *E. coli* O157:H7 contamination was statistically significant ($p = 0.013$). Similarly, *E. coli* O157:H7 contamination level of beef in association with Hawassa and Yirgalem was statistically significant ($p = 0.044$). In contrast to the above, there were statistically insignificant associations that observed in between some variables and their *E. coli* O157:H7 contamination level. For instance, beef from butcher shops showed a higher contamination rate than those from abattoirs, environmental swabs shown greater contamination rate than meat swab and hand swab, and among fish samples, *E. coli* O157:H7 contamination was notably higher in Nile tilapia (*Oreochromis niloticus*) than in African catfish (*Clarias gariepinus*) and contamination level was higher in fish skin swab than their muscle swab. The study also assessed the antimicrobial susceptibility of the *E. coli* O157:H7 isolates, which demonstrated complete susceptibility to several antibiotics, including ciprofloxacin, nalidixic acid, chloramphenicol, cephalothin, ceftazidime, ceftriaxone, gentamycin, and tetracycline. However, all isolates exhibited 100% resistance to clindamycin, with high resistance to ampicillin (81.8%) and moderate resistance to streptomycin (45.5%). Additionally, multidrug resistance (MDR) was observed in 45.45% of isolates, with an MDR index of 0.273, although none of the isolates showed extended-spectrum beta-lactamase (ESBL) production.

Based on the above conclusion, the following recommendations can be forwarded:

- ✓ To conduct further research on detected *E. coli* O157:H7 isolates, focusing on their molecular relatedness to *E. coli* O157:H7 strains isolated from stool samples of diarrheic patients of those study areas.

- ✓ To investigate the primary sources of contamination in Lake Hawassa, specifically determining whether the fish contamination by *E. coli* O157:H7 was due to animal feces, human waste, or other environmental factors.
- ✓ To utilize advanced molecular techniques, such as whole genome sequencing, to provide deeper insights into the genetic characteristics of *E. coli* O157:H7 isolates and their transmission dynamics.
- ✓ To implement continuous and systematic surveillance programs to monitor antimicrobial resistance patterns in *E. coli* O157:H7 isolates, guiding the prudent use of antimicrobials in veterinary and human medicine.
- ✓ To expand future research to include larger sample sizes, various animal origin food types and more diverse geographical areas to gain a broader understanding of *E. coli* O157:H7 occurrence and antimicrobial resistance in different food types and ecosystems.
- ✓ To establish a microbial isolate database to support comparative studies and enhance regional capacity for microbiological research.

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8. APPENDICES

I. Data collecting sheet

Tables in appendix 1: Data collection sheet format

Date	Code	Study area	Food type/ beef/milk/ fish	Specimen type	Sample Source	Fish species	Remark

II. Laboratory data registration sheet

Tables in appendix 2: Laboratory data recording sheet format

No	Sample Code	Result Part														
		Macconkey Agar	Subculture In Macconkey	Gram Stain	Catalase Test	Triple Sugar Iron Test				Sulfide Indole Motility Test			Simmons Citrate Test	E.Coli Result	Sorbitol Macconkey Agar Test	Molecular Test *E. coli O157*:H7
						Slant Color	Butt Color	H_2S Production	Gas Production	Sulfide Production	Indole Test	Motility Test				

III. Antimicrobial susceptibility test data recording sheet

Tables in appendix 3: Antimicrobial susceptibility test data recording sheet format

No.	Code	Drugs tested and the zone of inhibition measures in mm										
		AMP10	CIP5	C30	CTR30	CD2	KF30	S10	T30	NA30	CN10	CAZ30

IV. Primary Biochemical tests procedures

a. Catalase Test

a) Place a small amount of bacterial colony from a nutrient agar plate onto a clean glass slide.
b) Add 1-2 drops of 3% hydrogen peroxide (H_2O_2) onto the bacterial smear.
c) Observe for the production of bubbles.

- Interpretation:
  - ✓ Positive Result: Immediate bubbling or froth formation due to the release of oxygen gas (O_2) from the breakdown of H_2O_2. This indicates the presence of catalase enzyme, which breaks down hydrogen peroxide into water and oxygen.
  - ✓ Negative Result: No bubbles form, indicating the absence of the catalase enzyme.
  - ✓ *E. coli* O157:H7 is catalase-positive.

b. Gram Stain

a) Prepare a bacterial smear on a glass slide and heat-fix it.
b) Apply crystal violet stain for 1 minute, then rinse with water.
c) Add iodine solution for 1 minute, then rinse with water.
d) Decolorize with 95% ethanol for 10-15 seconds and rinse.
e) Counterstain with safranin for 30 seconds, then rinse with water.

f) Blot the slide dry and observe under a microscope.

- Interpretation:
  - Gram-Positive: Bacteria retain the crystal violet-iodine complex and appear purple.
  - Gram-Negative: Bacteria lose the crystal violet stain and take up the safranin counterstain, appearing pink/red.
  - *E. coli* O157:H7 is Gram-negative (appears pink under the microscope).

V. Laboratory Media'sUsed, Their Description and Procedures Undertaken

1. Sorbitol MacConkey Agar (SMAC) (HiMedia)

1. Purpose: SMAC is used for the selective isolation of *Escherichia coli* O157:H7 by differentiating sorbitol-fermenting strains (which are usually non-pathogenic) from sorbitol non-fermenting strains like *E. coli* O157:H7
2. Composition:Peptone: 20 g, Sorbitol: 10 g, Bile salts: 1.5 g, Sodium chloride (NaCl): 5 g, Neutral red (indicator): 0.03 g, Crystal violet: 0.001 g, and Agar: 15 g
3. pH: 7.1 ± 0.2
4. Preparation:
   - ✓ Weighing: Dissolve 51.5 g of SMAC powder into 1 liter of distilled water.
   - ✓ Sterilization: Autoclave the medium at 121°C for 15 minutes.
   - ✓ Cooling: Let the autoclaved medium cool to about 50°C.
   - ✓ Pouring: Pour the medium into sterile Petri dishes (approximately 20-25 mL per plate) and allow it to solidify at room temperature.
5. Procedure in the Experiment:
   - ✓ Inoculation: Streak the food or environmental samples (e.g., meat swabs, environmental swabs) directly onto the surface of the SMAC plates.
   - ✓ Incubation: Incubate the inoculated plates at 37°C for 18-24 hours under aerobic conditions.
6. Colony Appearance and Interpretation: Sorbitol-non-fermenting colonies (presumptive *E. coli* O157:H7) appear colorless or pale. This occurs because they cannot ferment sorbitol. Sorbitol-fermenting colonies (pink/red) indicate non-pathogenic *E. coli* strains

7. Storage and Shelf Life:
   - Prepared Plates: Store SMAC plates at 4°C for up to two weeks. Avoid exposure to light and moisture, which can degrade the medium.
   - Powder Form: SMAC medium powder can be stored at room temperature in a dry, dark environment for up to one year.

2. MacConkey Agar (MAC) (HiMedia)

1. Purpose: MacConkey Agar is a selective and differential medium used to isolate gram-negative enteric bacteria and differentiate them based on lactose fermentation. It is commonly used to isolate *Escherichia coli* and other enteric pathogens from clinical and food samples. The presence of bile salts and crystal violet inhibits the growth of gram-positive organisms.
2. Composition: Peptone: 20.0 g, Lactose: 10.0 g, Bile Salts: 1.5 g, Sodium Chloride: 5.0 g, Neutral Red: 0.03 g, Crystal Violet: 0.001 g, Agar: 15.0 g,
3. pH: 7.1 ± 0.2 at 25°C
4. Procedure:
   - Preparation: Dissolve 51.5 grams of the medium in 1 liter of distilled water. Heat to boiling with frequent agitation until the medium is completely dissolved. Autoclave at 121°C for 15 minutes.
   - Pouring: After sterilization, allow the medium to cool to 45-50°C and pour into sterile Petri dishes.
   - Inoculation: Aseptically inoculate the test sample on the surface of the agar. Streak the sample to isolate colonies.
   - Incubation: Incubate plates aerobically at 37°C for 24-48 hours.
5. Interpretation of Results:
   - Lactose Fermenters: Bacteria that ferment lactose produce acid, which lowers the pH and results in pink to red colonies. The neutral red indicator turns red at lower pH.
   - Non-Lactose Fermenters: Bacteria that do not ferment lactose produce colorless or pale colonies.
   - Selective Nature: The bile salts and crystal violet inhibit the growth of gram-positive bacteria, thus selectively isolating gram-negative organisms.

6. Storage and shelf Life:
   - Prepared Plates: Store MAC plates at 4°C for up to two weeks. Avoid exposure to light and moisture, which can degrade the medium.
   - Powder Form: MAC medium powder can be stored at room temperature in a dry, dark environment for up to one year.

3. Mueller-Hinton Agar (HiMedia)

1. Purpose: A solid medium specifically used for antimicrobial susceptibility testing (e.g., Kirby-Bauer disk diffusion test).
2. Composition: Beef extract: 2g, Acid hydrolysate of casein: 17.5g, Starch: 1.5g and Agar: 17g
3. pH : 7.3 ± 0.1
4. Preparation Instructions:
   - ✓ Dissolve 38g of Mueller-Hinton agar powder in 1 liter of distilled water.
   - ✓ Bring to a boil to dissolve the medium completely.
   - ✓ Sterilize by autoclaving at 121°C for 15 minutes.
   - ✓ Cool to 50°C and pour into sterile Petri dishes (approximately 25 mL per plate).
5. Usage in the Experiment
   - ✓ Inoculation: Spread bacterial culture evenly across the surface of the plate for disk diffusion tests.
   - ✓ Incubation: Incubate at 35°C for 16-18 hours.
   - ✓ Result Interpretation: Measure zones of inhibition around antibiotic disks to determine susceptibility.
6. Storage: Store plates at 4°C. Plates can be used for several weeks if properly stored.

4. Triple Sugar Iron (TSI) Agar (HiMedia)

1. Purpose: This medium is used to differentiate Enterobacteriaceae based on their ability to ferment sugars (glucose, lactose, and sucrose) and produce hydrogen sulfide.
2. Composition: Peptone: 10 g, Glucose: 1 g, Lactose: 10 g, Sucrose: 10 g, Sodium thiosulfate: 0.3 g, Ferric ammonium citrate: 0.2 g, Phenol red (pH indicator): 0.024 g, and Agar: 15 g

3. pH: 7.3 ± 0.2
4. Preparation Instructions:
   - ✓ Dissolve 65g of TSI agar powder in 1 liter of distilled water.
   - ✓ Boil to completely dissolve the medium, then sterilize by autoclaving at 121°C for 15 minutes.
   - ✓ Cool the medium to 50°C and dispense into sterile tubes as slants (allow the medium to solidify at an angle to create a slant with a deep butt).
5. Usage in the Experiment:
   - ✓ Inoculation: Using a sterile needle, stab the butt of the TSI agar and streak the surface of the slant with the sample.
   - ✓ Incubation: Incubate at 37°C for 18-24 hours.
6. Result Interpretation:
   - Yellow slant/butt: Fermentation of lactose and/or sucrose.
   - Yellow butt/red slant: Glucose fermentation only.
   - Black precipitate: Hydrogen sulfide (H_2S) production.
   - Gas bubbles/cracks in agar: Gas production.
7. Storage: Store TSI agar tubes at 4°C. Prepared slants can be stored for up to several weeks

5. SIM Medium (Sulfur, Indole, Motility) Agar (HiMedia)

1. Purpose: Used to test for hydrogen sulfide production, indole production, and bacterial motility.
2. Composition: Peptone: 30g, Sodium thiosulfate: 0.2g, Ferrous ammonium sulfate: 0.2g, and Agar: 3g
3. pH: 7.1 ± 0.2
4. Preparation Instructions:
   - Dissolve 30g of SIM medium powder in 1 liter of distilled water.
   - Sterilize by autoclaving at 121°C for 15 minutes.
   - Cool to 50°C and dispense into sterile test tubes.
5. Usage in the Experiment:

- Inoculation: Using a straight needle, inoculate by stabbing the center of the medium with the sample.
- Incubation: Incubate at 37°C for 24-48 hours.

6. Result Interpretation:
   - Hydrogen Sulfide Production: Black precipitate along the stab line indicates H_2S production.
   - Indole Production: Add Kovac's reagent after incubation. A red ring at the top of the medium indicates a positive indole test.
   - Motility: Diffuse growth radiating away from the stab line indicates motility.
7. Storage: Store SIM medium tubes at 4°C for up to several weeks.

6. Simmons Citrate Agar (HiMedia)

1. Purpose: Used to determine whether an organism can use citrate as its sole carbon source.
2. Composition: Sodium citrate: 2g, Ammonium dihydrogen phosphate: 1g, Dipotassium phosphate: 1g, Sodium chloride: 5g, Magnesium sulfate: 0.2g, Bromothymol blue (indicator): 0.08g, and Agar: 15g
3. pH: 6.9 ± 0.2
4. Preparation Instructions:
   - Dissolve 24.28g of Simmons citrate agar powder in 1 liter of distilled water.
   - Sterilize by autoclaving at 121°C for 15 minutes.
   - Pour into sterile Petri dishes or tubes as slants and allow to solidify.
5. Usage in the Experiment:

- Inoculation: Lightly streak the surface of the slant with the sample.
- Incubation: Incubate at 37°C for 24-48 hours.

6. Result Interpretation: A color change from green to blue indicates positive citrate utilization.
7. Storage: Store prepared slants or plates at 4°C for up to several weeks.

7. Trypton Soya Broth (TSB) (HiMedia)

1. Purpose: A general-purpose enrichment medium for cultivating a wide range of organisms, including *E. coli* O157:H7
2. Composition:Pancreatic digest of casein: 17g, Enzymatic digest of soybean: 3g, Sodium chloride: 5g, Dextrose: 2.5g, and Dipotassium phosphate: 2.5g
3. pH: 7.3 ± 0.2
4. Preparation Instructions:
    - ✓ Dissolve 30g of TSB powder in 1 liter of distilled water.
    - ✓ Sterilize by autoclaving at 121°C for 15 minutes.
2. Usage in the Experiment:
    - ✓ Inoculation: Inoculate the broth with a loopful of the sample.
    - ✓ Incubation: Incubate at 37°C for 18-24 hours.
    - ✓ Observation: Growth is indicated by turbidity.
6. Storage: Store prepared broth at 4°C for up to a few weeks

8. Buffered Peptone Water (BPW) (**HiMedia**)

1. Purpose: Non-selective pre-enrichment medium for the recovery of *E. coli* O157:H7and other bacteria from food samples.
2. Composition: Peptone: 10g, Sodium chloride: 5g, Sodium phosphate (dibasic): 3.5g, and Potassium phosphate (monobasic): 1.5g
3. pH: 7.0 ± 0.2
4. Preparation Instructions:
    - ➢ Dissolve 20g of BPW powder in 1 liter of distilled water.
    - ➢ Sterilize by autoclaving at 121°C for 15 minutes.
5. Usage in the Experiment:
    - ➢ Inoculation: Add food or environmental samples to the BPW and incubate.
    - ➢ Incubation: Incubate at 37°C for 18-24 hours.
    - ➢ Observation: The presence of bacteria is indicated by growth (turbidity).
6. Storage: Store at 4°C for up to a few weeks.

9. Nutrient Agar (HiMedia)

1. Purpose: General-purpose solid medium used for the cultivation of a wide variety of non-fastidious microorganisms, including *E. coli*.
2. Composition: Peptone: 5g, Beef extract: 3g, Sodium chloride: 5gand Agar: 15g
3. pH : 7.0 ± 0.2
4. Preparation Instructions:
    - ✓ Dissolve 23g of nutrient agar powder in 1 liter of distilled water.
    - ✓ Bring to a boil to dissolve the medium completely.
    - ✓ Sterilize by autoclaving at 121°C for 15 minutes.
    - ✓ Cool the medium to around 50°C and pour into sterile Petri dishes (approximately 20 mL per plate).
    - ✓ Allow plates to solidify at room temperature.
5. Usage in the Experiment
    - ✓ Inoculation: Streak the sample onto the surface of the solidified agar plate.
    - ✓ Incubation: Incubate at 37°C for 18-24 hours.
    - ✓ Result Observation: Growth of bacterial colonies is visible on the agar surface.
6. Storage: Store prepared plates at 4°C in airtight containers. Plates can be stored for up to two weeks.

10. Nutrient Broth (HiMedia)

1. Purpose: A general-purpose liquid medium for the growth and enrichment of a wide range of non-fastidious microorganisms.
2. Composition: Peptone: 5g, Beef extract: 3g, and Sodium chloride: 5g
3. pH: 7.0 ± 0.2
4. Preparation Instructions:
    - ✓ Dissolve 8g of nutrient broth powder in 1 liter of distilled water.
    - ✓ Sterilize by autoclaving at 121°C for 15 minutes.
    - ✓ Dispense into sterile tubes or flasks for use.
5. Usage in the Experiment:
    - ✓ Inoculation: Inoculate the broth with the sample using a loopful or swab.
    - ✓ Incubation: Incubate at 37°C for 18-24 hours.

6. Result Observation: Growth is indicated by turbidity or cloudiness in the broth.
7. Storage: Store prepared broth at 4°C. Use within a few weeks.

11. Brain Heart Infusion (BHI) Broth(HiMedia)

1. Purpose: A rich nutrient medium used for cultivating fastidious organisms, including *E. coli* , and for the preparation of bacterial suspensions.
2. Composition: Calf brain infusion: 200g, Beef heart infusion: 250g, Peptone: 10g, Sodium chloride: 5g, and Glucose: 2g
3. pH : 7.4 ± 0.2
4. Preparation Instructions
   - ✓ Dissolve 37g of brain heart infusion broth powder in 1 liter of distilled water.
   - ✓ Sterilize by autoclaving at 121°C for 15 minutes.
   - ✓ Dispense into sterile tubes or flasks.
5. Usage in the Experiment
   - ✓ Inoculation: Inoculate the broth with the sample.
   - ✓ Incubation: Incubate at 37°C for 18-24 hours.
   - ✓ Result Observation: Turbidity or cloudiness in the broth indicates bacterial growth.
6. Storage: Store at 4°C for several weeks.

12. Glycerol 20% Solution

1. Purpose: Used as a cryoprotectant for preserving bacterial cultures at low temperatures.
2. Composition: Glycerol: 200 mL and Sterile distilled water: 800 mL
3. Preparation Instructions:
   - ✓ Mix 200 mL of glycerol with 800 mL of sterile distilled water to prepare a 20% glycerol solution.
   - ✓ Sterilize the solution by autoclaving at 121°C for 15 minutes.
4. Usage:

- ✓ Preservation: Add equal volumes of bacterial culture and 20% glycerol solution to cryovials for long-term storage.
- ✓ Storage: Freeze at -80°C for long-term preservation.

5. Storage: Store the glycerol solution at room temperature for up to one year if sterile.

V. Molecular Detection procedures

A. DNA extraction procedure

1. Sample Preparation
    - Add 250 µL of the bacterial sample to a 1.5 mL microcentrifuge tube.
    - Centrifuge at 12,000 x g for 10 minutes to pellet the cells. Carefully discard the Supernatant, leaving the pellet intact.
2. Cell Lysis
    - Add 100 µL of 1x Tris-EDTA (TE) buffer to the pellet for buffering.
    - Introduce 20 µL of Proteinase K solution to facilitate protein digestion.
    - Freeze the tube at -80°C for 15 minutes to induce cellular damage, enhancing lysis efficiency.
3. Heat-Induced Lysis
    - After thawing, incubate the tube at 95–100°C for 15 minutes to lyse the cells. This step uses heat to disrupt cell walls, freeing the DNA.
4. RNase Treatment
    - Allow the tube to cool to room temperature. Add 2 µL of RNase A and incubate at 37°C for 15 minutes to remove RNA from the sample.
5. Centrifugation and DNA Collection
    - Centrifuge the tube at 13,000 x g for 3 minutes to separate cell debris. Carefully transfer the supernatant, containing the DNA, into a new sterile microcentrifuge tube.
6. Storage
    - Store the DNA extract at -20°C for long-term use or use immediately as a DNA template in further experiments.

B. Master mix preparation

1. Prepare reaction components
    - ✓ In a sterile microcentrifuge tube, add the following:
        - 2.5 µL of 10X PCR Buffer (with $MgCl_2$)
        - 0.5 µL of 10 mM dNTP mix
        - 1 µL of Forward Primer (10 µM)
        - 1 µL of Reverse Primer (10 µM)
        - 0.2 µL of Taq DNA Polymerase (5 U/µL)
        - Add nuclease-free water to reach a volume of 23 µL per reaction.
2. Aliquot Master Mix
    - Gently mix the master mix by pipetting up and down.
    - Dispense 23 µL of this master mix into each PCR tube or well of a PCR plate.
3. Add Template DNA
    - Add 2 µL of *E. coli* DNA template (10–100 ng) to each tube or well containing the master mix.
4. Briefly Spin and Store
    - Briefly spin the tubes or plate to collect all liquid at the bottom and ensure no bubbles.
    - Keep the tubes on ice if running PCR immediately, or store at 4°C for short-term use.

C. Multiplex PCR Amplification Steps

1. Initial Denaturation: 95°C for 3 minutes.
2. Cycle Conditions (34 cycles):
    - ✓ Denaturation: 95°C for 20 seconds
    - ✓ Annealing: 54°C for 1 minute
    - ✓ Extension: 72°C for 1 minute
3. Final Extension: 72°C for 7 minutes.
4. Held: 4°C until analysis.

D. Gel electrophoresis procedures

1. 1x TBE buffer preparation

i. 10.8 g of Tris-base and 5.5g of boric acid was dissolved in 805 mL distilled water.

ii. 4mL of 0.5M Ethylene diamine tetraacetic acid at pH = 8.0 was added to the above mixture.

iii. Finally, the volume was adjusted to 1 L by adding distilled water.

2. Preparation and loading of a 1.5% agarose gel

i. 4.5 g of agarose was added to a 500 mL measuring Pyrex flask containing 300 mL of 1xTBE buffer

ii. The mixture was then boiled with in a microwave until the agarose was fully dissolved.

iii. 10 µL of Ethidium bromide was added to the dissolved agarose at 45^{o}C and dissolved by

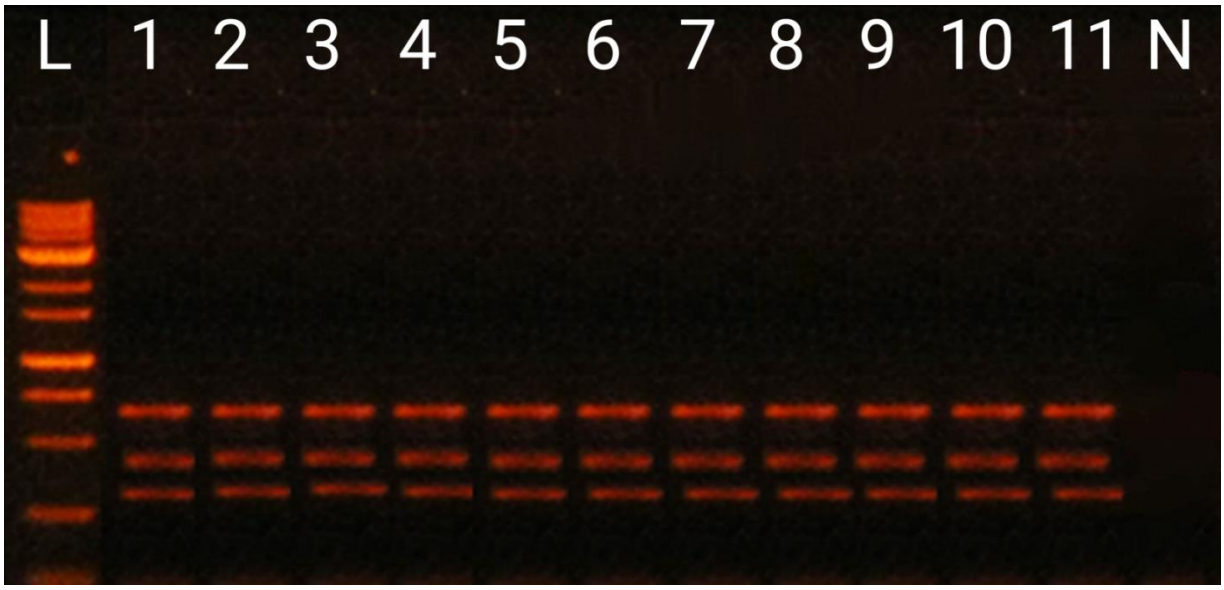
iv. Slowly shaking the flask after which it was poured onto the 25 cm gel making plate for gel casting.

v. Combs were gently removed, and the gel cast was placed into the gel tank and overlaid with1xTBE buffer fully covering the gel cast.

vi. Finally, the PCR product was loaded into the gel cast using a gel loading dye and 1 kb DNA ladder and allowed to run at 100 V for an hour.

vii. At the end, the DNA bands were visualized under the UV transilluminator and photographed.

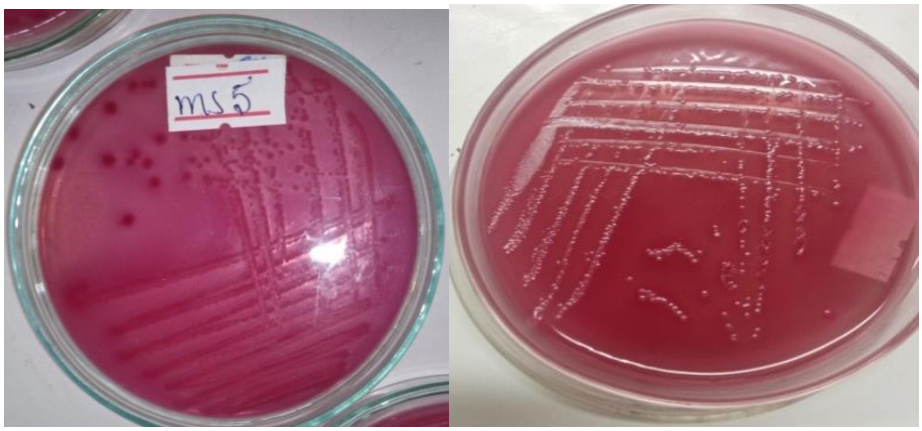
VI. Some Important Pictures during laboratory operations and data analysis



Figures in Appendix 1: Photograph picture of Gel electrophoresis result

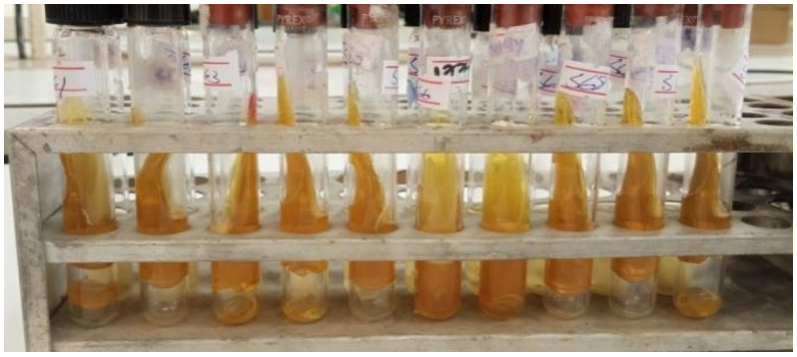
(Where L= 1 kb DNA ladder; lane 1 to 11= *E. coli* O157:H7 isolates; N= negative control (DNase/RNase water)

Figures in Appendix 2: When MacConkey agar was observed after 24 hours of incubation at 37^{o}c



Figures in Appendix 3: *E. coli* O157:H7 isolates in MacConkey agar

(Pink and circular colonies)



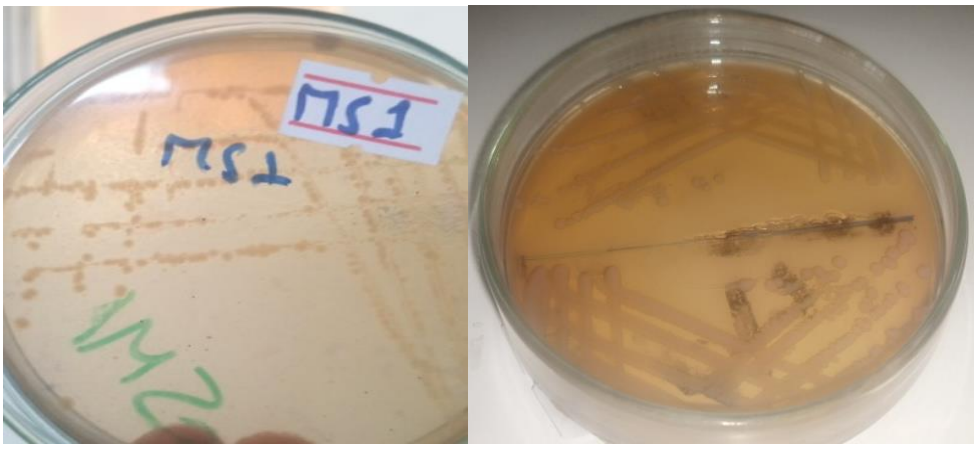
Figures in Appendix 3: TSI test result of *E. coli* O157:H7 isolates

(Yellow slant/yellow butt, Gas production and No blackening)

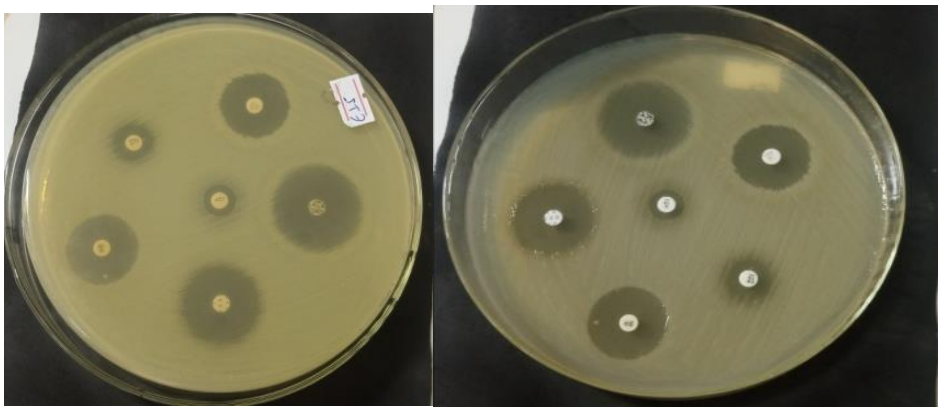
Figures in Appendix 5: SIM test result of *E. coli* O157:H7 isolates

(Sulphide production = Negative (no blackening); Indole test = Positive (Red ring on top of media; Motility = Positive (Cloudy media)

Figures in Appendix 6: Simmons Citrate agar result of *E. coli* O157:H7 isolates (Citrate negative; No color change of media, Media remains green)



Figures in Appendix 4: E. coli O157:H7 isolates in Sorbitol MacConkey Agar (Color-less colonies)



Figures in Appendix 8: Disc Diffusion test on Muller Hingtons media (Observed varying level of zone of inhibition against tested drugs

VAR00001 * VAR00002 Crosstabulation

			VAR00002		
			Negative	POSITIVE	Total
VAR00001	Beef	Count	118a	7a	125
		% within VAR00001	94.4%	5.6%	100.0%
		% within VAR00002	41.1%	63.6%	41.9%
	Fish	Count	60a	4a	64
		% within VAR00001	93.8%	6.2%	100.0%
		% within VAR00002	20.9%	36.4%	21.5%
	milk	Count	109a	0b	109
		% within VAR00001	100.0%	0.0%	100.0%
		% within VAR00002	38.0%	0.0%	36.6%
Total		Count	287	11	298
		% within VAR00001	96.3%	3.7%	100.0%
		% within VAR00002	100.0%	100.0%	100.0%

Each subscript letter denotes a subset of VAR00002 categories whose column proportions do not differ significantly from each other at the .05 level.

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)
Pearson Chi-Square	6.637[a]	2	.036	.037
Likelihood Ratio	10.292	2	.006	.011
Fisher's Exact Test	8.046			.013
N of Valid Cases	298			

a. 3 cells (50.0%) have expected count less than 5. The minimum expected count is 2.36.

Figures in Appendix 96: Screen shot picture of SPSS page of data analysis

VII. AUTHOR BIOGRAPHY

Workagegn Israel Asale, the author, was born on June 27, 1991, in the Wolaita zone of Southern Ethiopia to his father Israel Asale and his mother Birtanesh Tantu. He grew up in Bedessa, an administrative head town of Damot Woyde district of Wolaita zone, where he completed his education from kindergarten through grade 12. In 2009, he enrolled at Jigjiga University, Ethiopia, to pursue a degree in veterinary medicine. He studied there for six years, graduating in 2015 with distinction, earning a gold medal. Following his graduation, Workagegn worked as an animal health professional and also as a coordinator of animal health work process in the livestock and fishery department of Damot Woyde district of Wolaita zone for two and half years. In 2018, he joined the Southern Agricultural Research Institute, later renamed as Sidama Agricultural Research Institute, and has been serving as an animal health researcher for the past six years at the Hawassa Agricultural Research Center. His extensive experience and dedication to his field have been key to his contributions to Ethiopia’s agricultural research sector.